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Comparative analysis of auxinic compounds transporter ABCG37 and various polarly localized proteins in respect of secretion, trafficking and plasma membrane dynamics.

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Frequently used abbreviations:

ABCG37	ATP-Binding-Cassette (ABC), G subfamily
ARF	Adenosyl ribosylation factor
AUX1	AUXIN-RESISTANT1
BFA	Brefeldin A
BOR1	BORATE TRANSPORTER1
BOR4	BORATE TRANSPORTER4
CESA3	CELLULOSE SYNTHASE CATALITIC SUBUNIT3
CHX	cyclohexamide
2,4D	2,4-Dichlorophenoxyacetic acid
DIM	Detergent Insoluble Membrane
DMSO	Dimethylsulfoxide
EM	Electron Microscopy
e-	sodium azide, 2-deoxy-D-glucose
FM4-64	N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide
FRAP	Fluorescence Recovery After Photobleaching
GEF	Guanine nucleotide exchange factor
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
LatB	Latrunculin B
MS	Murashige and Shoog
NAA	Naphthalenacetic acid
1-NOA	1-napthoxy acetic acid
2-NOA	1-napthoxy acetic acid
NPA	1-N-naphtylphtalamic acid

PAA	phenylacetic acid (PAA)
PBA	Phthalamic acid
PDR	PLEIOTROPIC DRUG RESISITANT
PEN3	PENETRATION3
PID	PINOID
PIN	PIN-FORMED
PIP2	PUTATIVE INTRINSIC PROTEIN2
PIS1	POLAR AUXIN TRANSPORT INHIBITOR-INSENSITIVE1
PM	Plasma membrane
PP2A	Protein phosphatase 2A
REPP3	REGULATOR OF PIN POLARITY SMT STEROL METHYLTRANSFERASE1
TGN	Trans Golgi Network
TIBA	2,3,5-triiodobenzoic acid
TIRF	Total Internal Reflection Fluorescence
VIAFM	Variable Incidence Angle Fluorescence Microscopy

Scope and summary of the thesis:

Scope:

Plants as sessile organisms evolved a specific body structure and at the cellular level mechanisms that allow to survive under extreme environmental conditions. The body shape and subcellular processes are largely dependent on coordinated activity of a small molecule indole-3-acetic acid (IAA), auxin. Local gradients of IAA correlate spatiotemporally with such developmental events like embryogenesis, phyllotaxis, organ initiation or tropisms. Auxin maxima and minima are mostly mediated by auxin efflux carriers PIN's. Asymmetric distribution of these proteins determines the directional flow and facilitates the auxin gradient formation. Aberrations in apical or basal auxin-carriers localisation leads to severe developmental defects. Therefore, it is crucial to understand the mechanisms initiating and controlling polar proteins localisation.

Next to polarly distributed PIN's, there is a growing group of polarly localized proteins transporting hormones or nutrients placed at the outer lateral and inner lateral polar domains. In my work I was mostly focused on polarity and function of auxinic-like compounds transporter ABCG37/PIS1, which localises to outer lateral domain in epidermal cells. I tried to characterise the transporting function of this specifically localised protein and find the regulators and mechanisms determining polarity. In order to get a more global overview about components and processes controlling asymmetric distribution of proteins I have included other asymmetrically distributed proteins like ABCG36, BOR4 or BOR1 localised to outer- or inner-lateral domains, respectively.

Summary:

The role of auxin hormone and PIN transporters in plant development cannot be undermined. Therefore, in order to find auxin transport regulators or PIN polarity determining components, several auxin-related EMS screens have been performed. Dr. Kamil Růžička during his PhD found a 2,4D hypersensitive mutant which was identified as a *polar auxin transport inhibitor sensitive1* (*pis1*), *ABCG37/PIS1*. Our further investigations was aimed at identifying other possible functions of PIS1 as well as characterization of the phenotypes and protein localization. As a result we have found that *pis1* mutant roots show strongly enhanced sensitivity to auxinic compounds including synthetic auxins (2,4-D, 2-NOA) and inhibitors of auxin transport (1-NOA, NPA, PBA, TIBA), but didn't show any abnormal responses to IAA or PAA. Interestingly, *pis1* mutant showed increased sensitivity to indole-3-butyric acid (IBA) the endogenous auxin precursor, what shows another level of regulation for auxin homeostasis.

In order to have an idea about PIS1 protein localization we performed immunostaining of a primary root tip with anti-ABCG37. This staining allowed us to detect the ABCG37 signal exclusively at the outermost sides of lateral root cap and epidermal cells of the wild-type but not in *pis1-1* root tips. Next, we compared the localisation of ABCG37 and homologous ABCG36/PDR8/PEN3 transporter. Both proteins not only show almost identical localisation, but also displayed similar functions, transporting IBA, which was shown in root elongation assays and confirmed by transport measurements of radiolabeled [^{3}H]-IBA and [^{3}H]-IAA in protoplasts, yeasts and HeLa cells (**Chapter2**).

Basing on abovementioned observations, that PIS1 localizes to outer-lateral domain, we decided to study the trafficking to this novel polar domain. We analyzed the mechanism of polar delivery to the surface of the epidermal cells facing environment of

three different proteins BOR4, ABCG37, and PEN3 which transport nutrients and plant hormones or are required for pathogen defense, respectively. Visualization of these proteins and apical and basal cargos in a single cell demonstrates that the outermost cell side represents an additional polar domain in plant cells. To check the occurrence of the outer lateral domain in different cell types, we examined the *35S::GFP-ABCG37* transgenic line expressing ectopically the functional GFP-ABCG37 throughout all the cell types of the root. Next, we tested how newly synthesised proteins reach the plasma membrane. Fluorescence Recovery After Photobleaching (FRAP) experiments revealed that the polar localisation of BOR4-GFP, GFP-ABCG37 or PEN3-GFP is achieved in a polar fashion at the earliest detectable recovery stages. In order to further test the secretion and recycling processes we used the fungal toxin Brefeldin A (BFA), that targets a subgroup of vesicle budding regulators ARF GEFs. BFA-treated lines indeed showed protein agglomerations. However in similar experiment including the inhibition of protein synthesis by cycloheximide the BFA bodies have been largely reduced. This observation suggests that the outer polar cargos are delivered to their polar domain by BFA-sensitive, ARF GEF-mediated polar secretion. To test the involvement of actin cytoskeleton in intracellular trafficking we performed the depolymerisation of actin filaments by Latrunculin B (LatB). This treatment did not visibly affect the outer lateral localization of BOR4-GFP, GFP-ABCG37 or PEN3-GFP and interestingly the limited intracellular aggregations were again mostly related to the secretion of the *de novo* synthesized proteins as demonstrated by their disappearance following cycloheximide treatment. Importantly, the outer polar localization does not require the known molecular components of the apical or basal targeting. Such as *gnom* ARF GEF, *axr4-1*, PINOID kinase and PP2A protein phosphatase did not affect the outer localizations of BOR4-GFP, ABCG37, and PEN3-

GFP. In summary, these data reveal that, outer polar targeting requires distinct molecular components than known apical and basal targeting pathways (**Chapter3**).

Initial observations concerning outer-lateral domain encouraged us to further investigate the processes of polarity establishment and maintenance not only at the outer-lateral but also at three others described in plants polar domains. Our first goal was to describe the protein lateral diffusion process which was so far described as a negligible factor in respect of influence on whole mechanism of polarity maintenance. By FRAP experiments and recovery measurements in time we estimated that the lateral diffusion rate differ between different polar and nonpolar markers. We have shown that two close PIN homologues, PIN1 expressed in stele and PIN2 expressed in epidermal cells have different lateral diffusion rates. PIN2 shows especially low diffusion, wheras PIN1 shows comparable diffusion rates with the apolar PIP2. This suggests, that a low diffusion rate does not correlating with polarly localized proteins, but most likely with clustering and specific cargo retention. Subsequently, we have studied the protein secretion to the plasma membrane. Basing on total FRAP results showing polar cargo delivery in early stages after bleaching, total recovery rates or experimentaly obtained laterl diffusion rates and theoretical assumption that specific sorting occurs at TGN, we have established new secretion model. Data implemented into computer simulations, showed that the preferentially polar secretion is necessary to initiate and maintain polar protein distribution. Data-based simulations were very closely resembling the real signal distribution during the live-imaging after photobleaching. Also the obtained recovery profiles and polarity indexes where similar to those obtained from experiments and calculations. Interestingly, the simulation of initially proposed nonpolar secretion trafficking, based on low diffusion, apolar secretion, low secretion rate, immediate and efficient polar recycling showed unrealistic recovery profiles and polarity index. In summary our results suggest that polar

secretion is a key regulator in polarity establishment and combined with polar recycling maintain the asymmetric protein distribution. Studies on several markers localizing to four defined polar domains suggest that PIN1, PIS1, PEN3 and BOR1 are preferentially polarly secreted to their specific domains. PIN2 which undergoes phosphorylation based modification shows different polarity index pattern, however it doesn't exclude the specific targeting of the protein. We also demonstate that all tested polar and non-polar markers are connected to the cell wall. However, the protein accumulation is much higher in case of polarly localized proteins and especially high in case of PIN2. This observation suggest that polarly localized and clustered proteins can be stabilize at the polar domains at the higher extend than nonclusterd and nonpolar. Finally we show that polar protein localization is not the same at different developmental stages. PIS1 and PEN3 show polarity transition between basal and outer-lateral domain during embryogenesis and in emerging lateral roots. This suggests that the differential protein polar targeting depending on developmental stage (**Chapter4**).

CHAPTER1

Multi-dimensional mechanisms determine polar protein localization

Łukasz Łangowski and Jiří Friml

Author's Contribution: ŁŁ and JF discussed and wrote the manuscript.

Introduction:

Ranging from unicellular organisms to complex multicellular eukaryotes one can observe that cellular polarity is a fundamental property across all kingdoms of living organisms. At the single cell level either it can be defined as a three-dimensional structural asymmetry or asymmetry in localization of intracellular molecules. Physical asymmetry, including that of extracellular matrix and heterogeneity of plasma membrane, may results in precise recruitment or retrieval of membrane proteins. While, asymmetry in protein distribution can be achieved by specific trafficking of the cargo to a certain polar domain [Dhonukshe 2005]. Both asymmetries interrelate with each other allowing in adequate organization of the molecules and prompt responses to internal and external cues to reorganize. Moreover, polarity is intertwined with all other aspects of cell biology, including differentiation, signaling, cytoskeletal organization, migration and division. [Mostov 1992, 2000]. These processes are deranged in many serious defects and diseases [Stein 2002]. During development, polarized traffic pathways are modified to accommodate the specific needs of individual cell types, as well as aid the organization of cells into tissues and organs; and only now, the principles of these modifications are emerging [Mostov 2003].

In plants, the complexity of the polar targeting machinery seems to be more pronounced than in animal cells. Apical, basal, inner lateral and outer lateral polar localizations of different plant proteins comparing to predominant apical and basolateral domains of animal cell shows how distinct are both systems already at the level of polar domain organisation. Moreover, in plants at the level of molecular regulators one will not find a single orthologue of well established cell polarity determinants in animals [Geldner 2009]. Besides the different number of polar domains and entirely different molecular machinery, there are several fundamental differences between animal and plant cell

polarity which are related with the retention of cargoes at polar domains. In animals the apical and basolateral domains are separated by tight junctions, which provide a barrier limiting movement of cargos between the domains [Shin 2006]. In contrast, in most plant cells a comparable structure is missing with exception of the so-called Casparian stripe that separates inner lateral and outer lateral domains in endodermal cells. In addition, in plants a cell wall seems to be a prominent player stabilizing protein polar localization. Besides those differences, there is another level of polarity regulation and maintenance, a heterogeneity of plasma membrane, which seems to play important role as well in animals as in plants [Malínská 2011].

In this review we focus on three major factors determining and maintaining polarity in plants: (i) Polarized traffic, focusing on secretion and highlighting importantce of recycling; (ii) and lateral diffusion of proteins within the plasma membrane; and (iii) plasma membrane heterogenity, which together with the presumptive connections between the plasma membrane and extracellular matrix is a so far underappreciated mechanism for specific cargo retention.

Secretion, diffusion, recycling:

It has been reported that in metazoa the polarized trafficking is based on three major processes [Nelson 2001]. First, newly synthesized proteins are transported through the Golgi Apparatus to the *trans*-Golgi network (TGN), where the sorting occurs into carriers that deliver them to apical or basolateral surfaces [Jacob 2001, Kreizer 2003]. Second, some proteins delivered to the cell surface are selectively retained, what very often occurs via an interaction of their carboxyl termini with PDZ-domain [Harris 2001], a common motive of 80-90 amino acids present in post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1), which were

first discovered to share the domain [Kennedy 1995]. Third, components that are not retained at the surface are rapidly endocytosed and delivered to early endosomes, from where they can be recycled back to the cell surface, transferred to late endosomes or transported across the cell and delivered to the opposite surface, a process known as transcytosis [Mostov 2000, 2003]. Importantly, Mostov and coleagues reported that all epithelial cells use biosynthetic sorting from the TGN and selective recycling/transcytosis to deliver the cargo to specific surface. However, the relative importance of these processes varies with the cell type. Moreover, the flux through the endocytic pathway is approximately ten times greater than through the biosynthetic pathway, indicating that selective recycling/transcytosis is essential for steady-state polarity [Bomsel 1989, Mostov 2003].

In plants the mechanisms controlling sorting, recycling and maintaining polarity are still poorly understood. However some initial insights have already been provided. It has been proposed that non-polar protein secretion combined with low lateral protein diffusion rate and especially efficient polar endocytic recycling largely contribute to establish and maintain asymmetric protein distribution [Donukshe 2008]. Recent results based on more refined quantitative imaging combined with computer simulations Kleine-Vehn MSB]; confirmed a crucial importance of endocytic recycling but showed that an important contribution of polar secretion cannot be excluded.

FRAP analysis which have been done on five different polar markers and one apolar, showed recovery range between 55-80% whitin 3h time that doesn't align with the half life time of the regular plasma membrane proteins in animals (20h) [Bomsel 1989]. Moreover, the signal intensity at polar and nonpolar domains, within the cell, after 30 min of recovery, is more pronounced at the polar domain, suggesting two possible scenarios: (i) Newly synthesized proteins are specifically sorted and preferentially polarly secreted to the

PM; (ii), the biosynthetic sorting and secretion are random, followed by rapid endocytosis and immediate polar sorting and very rapid recycling. Yet, it has been shown that PIN2-EosFP internalized following treatment with recycling inhibitor brefeldin A (BFA), needs more than 50 min to be recycled back to PM after BFA removal, a complete recovery of PM localization takes approximately 100 min [Dhonukshe 2007]. This result suggests, that polar recycling is not a rapid process, however precise endogenous dynamics cannot be convincingly inferred from experiments based on BFA treatments, in particular because molecular targets of BFA, the ARF GEFs are involved not only in recycling but also in endocytosis [Naramoto 2010] and thus most likely distort the whole dynamics of endocytic recycling. Nonetheless, taking this into account the recent data on the FRAP-based polarity establishment dynamics (Chapter 4), we revised the model of polarity establishment and maintenance and gave more importance to polar secretion process. This implies that polar sorting must occur not only during endocytic recycling but also somewhere along the secretory route that delivers cargos to the specific plasma membrane surface. Once the cargos are within the polar domain, the proteins are laterally diffusing within the plasma membrane with different speeds depending on the type of protein. Some proteins are specifically binding to other proteins or are retained in microdomains that slow down their movement (this aspect will be discussed in detail later on). At some point laterally diffusing cargos reach the boundaries of their polar domains. At that time the clathrin-dependent endocytic recycling plays a crucial role [Dhonukshe 2007, Kleine-Vehn 2011]. Cargos, exemplified by PIN proteins are internalized into presumptive Early Endosomes (EE), which in plants seem to be equivalent to the TGN [Dettmer 2006, Lam 2007, Grunewald 2010]. TGN/EE is the place where the biosythetic and recycling routes meet, what in consequence leads to polar recycling of the endocytosed cargos and continuous re-establishment of polar distribution.

The limiting factors to study and precisely dissect secretion or recycling are tools. It is difficult to obtain transgenic lines with GFP-tagged expression reflecting the natural expression levels and the possible effect of the tag on the dynamical behavior is always an issue. Too high expression may lead to saturation of the system and alterations in quality and dynamics of sorting and secretion. If these are inducible lines, then it seems necessary to assess the RNA or protein level during optimalisation of the experimental system. In fact, conditional overexpression was used to dissect secretion and recycling processes. The inducibly expressed PIN1 and PIN2 were initially apolarly secreted and later polarized at specific polar domain [Dhonukshe 2008; Dhonukshe 2010]. In case of PIN1 it was inducible overexpression of PIN1 in epidermal cells. 60 min after induction the protein was localized apolarly, one hour later preferentially to the outer lateral domain. Finally, 180 min after induction the protein is polarly localized, however, the time needed for the polarization far exceeds the time inferred from the FRAP experiments that showed contrasting results with newly synthesized PIN1 appearing specifically on the polar side from early stages of it recovery [Dhonukshe 2008, Chapter4]. Follow-up experiments using epidermis-specific conditional overexpression confirmed apolar secretion and endocytic recycling-based polarization [Dhonukshe 2010]. PIN1 localized apolarly after 2h and polarized after 6h of induction, PIN2 showed apolar localization after 5h and apical localization after 10h of induction. Thus the dynamics of polarization was again strikingly different that that inferred from FRAP-based observations (Chapter 4). These observations might be the result of saturation of the system with overexpressed cargoes and consequent mis-sorting and mis-secretion of the polar cargos. Inducible systems are attractive, however, for specific questions related to trafficking they may be, without special controls, misleading and introducing artifacts. The best approach to address the problem of trafficking dynamics would be the visualization of photoconverted pool of the proteins and

tracking the trafficking to the polar domain. To properly analyze and quantify secretion, we are still missing specific tools blocking the endocytosis without disruption of retrograde transport and sorting to the plasma membrane or vacuole. Thus, given the still limited tools and contradictory observations from different experimental designs, it is difficult to exactly delineated the relative contributions of polar secretion for overall polarity establishments, however, it seems obvious that, as in animals, in plants this process plays a so far unappreciated role in polarity establishment.

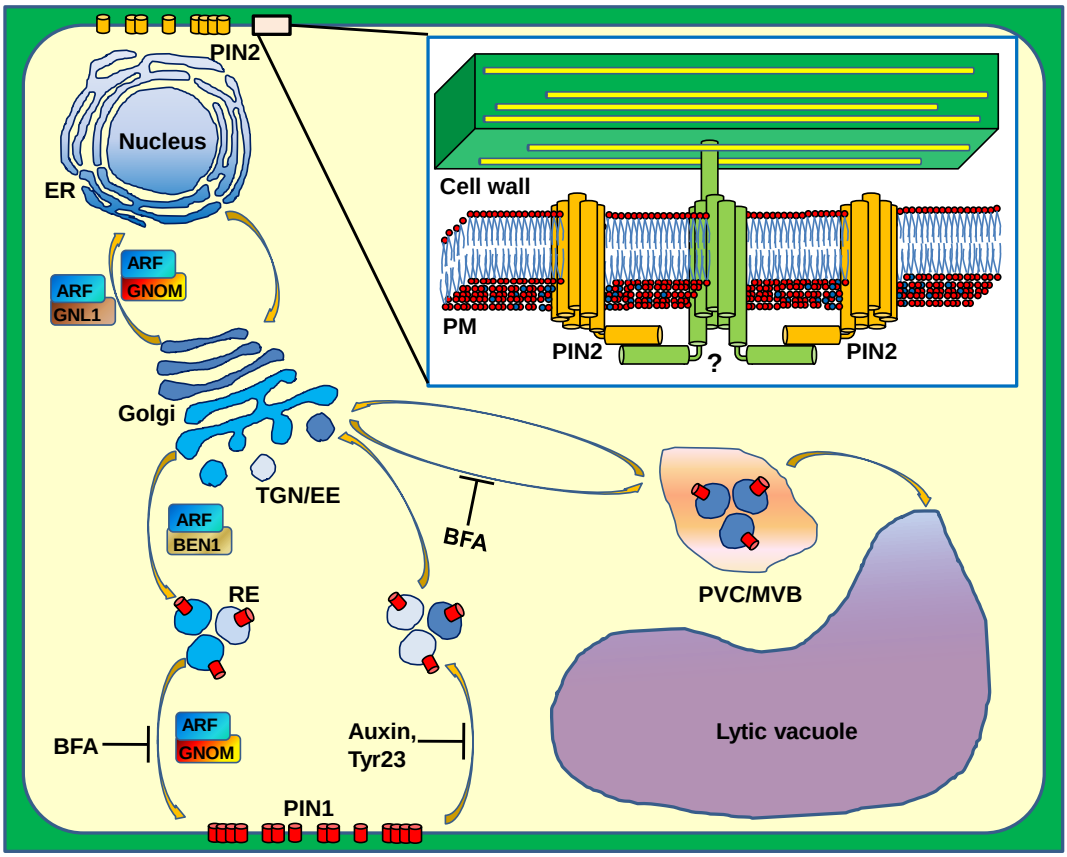

Figure 1. Endomembrane system and plasma membrane association with the cell wall.

Biosynthesized proteins starts the endomembrane cycling at ER, where they undergo folding and glycosylation. Further these proteins are transported to Golgi Apparatus, which in plants is organized in stacks [Jürgens 2004]. Each stack consists of distinct cisternae arranged from the *cis* to the *trans* side. Proteins moving from *cis* to *trans* cisternae undergoes further modifications ending up in the *trans*-Golgi (TGN). At TGN the crucial event for protein asymmetric distribution occurs. Proteins are either sorted to the cell surface or targeted to the vacuole [Viotti 2010]. After sorting process cargoes enter specific trafficking pathway reaching proper polar domains. Proteins freely diffusing within the

plasma membrane can associate with putative stabilizing molecules into clusters, which can be further stabilized by interaction with cell wall anchored proteins. At some point proteins get endocytosed with the membrane into vesicles typically coated by clathrin. These vesicles fuse with the endosomes, which in plants seem to be equivalent with TGN, where the sorting and recycling back to the plasma membrane occur [Dettmer 2011, Lam 2007].

Cell wall, Casparian Strip and Tight Junction:

In animals the asymmetric protein distribution is not only based on sophisticated intracellular processes like specific sorting, polar secretion and recycling which initiates and determine cell polarity but also on a "primitive" physical barrier, a tight junction [Yu 2008]. Tight junction scrupulously studied in *Drosophila melanogaster* and mammals is a selective barrier in epithelial cells in lungs or guts, that prevents the free diffusion of soluble molecules and membrane components through the intercellular space forming tight seals between distinct polar domains [Iden 2008; Assemat 2008]. Plants evolved structurally different but functionally similar diffusion barrier a ligno-suberic band, called Casparian Stripe [Caspary 1865/66]. This highly localized cell wall deposition in the transversal and anticlinal walls of the cell, which surrounds the cell like a belt, separates inner and outer polar domains in endodermal cells. The Casparian Strip tightly coordinated with respect to neighboring cells in a broader context controls the nutrient exchange between soil environment and root interior [Alassimone 2010; Roppolo 2011]. Casparian strip is a good example in plants for specific protein and material secretion, formation of the stripe is asymmetric and show the dynamic relation between cell wall and plasma membrane and proteins [Grebe 2011]. However, the Casparian strip is present only in endodermal cells, so in most cell types, other mechanisms assuring separation of polar domains must be present.

Secretion, protein lateral diffusion and polar recycling are prominent but not sufficient players in complex and multidimensional cellular polarization. Recently, it has been shown

that mutants defective in cell wall synthesis display PIN polarity transition from the basal to the apical polar domain, revealing the possible role of the extracellular matrix in polar distribution of cargos [Feraru 2011]. Basally localized PIN2::PIN1-HA introduced in *pin2* mutant shows agravitropic root phenotype. In the mutant screen designed to find determinants of basal localization, mutants partially or fully complementing an agravitropic growth were recovered; one of them, cellulose synthase mutant, *repp3* (*regulator of pin polarity3*) [Feraru 2011]. Mutation in this gene which is also known as a catalytic subunit 3/constitutive expression of VSP1/isoxaben resistant 1/ectopic lignin 1 (CESA3/CEV1/IXR1/ELI1) [Richmond 2000; Ellis 2001; Scheible 2001; Delgado 2003], leads to apical localization of PIN2::PIN1-HA [Feraru 2011]. This phenomenon suggest, that the cell wall is an important factor in polarity establishment and maintenance. However, proteins localizing to the apical or lateral polar domains in the same cells did not show any defects in polar distribution suggesting that CESA3 may play a specific role in stabilizing basal polar localization. The high number of available cell wall synthesis genes raises a possibility for functional redundancy of cellulose synthesis genes accounting for no obvious defects in polar distribution of apical and lateral cargos in single mutant backgrounds. It might also reflect that retention specifically at the basal polar domain is more strictly dependent on the cell wall composition.

A relatively crude test probing the role of extracellular matrix in polarity maintenance was protoplasting of the root cells by digestion of the cell wall. Various polarly localized markers defining apical, basal, outer-lateral and inner-lateral domains rapidly loose polar distribution after digestion of the entire cell wall [Feraru 2011; Chapter 4]. One can naturally debate to which extent are context-free and signaling-deprived cells still able to maintain the polarity mechanisms but these observations showed that

extracellular matrix and associated processes are absolutely required for maintenance of cell polarity.

To further investigate the role of cell wall in polarity maintenance, plasmolysis experiments on polar and nonpolar markers have been performed to physically detach the PM and the cell wall. Partial degradation of the cell wall revealed protein accumulation not only at shrinking plasma membrane but also to a large extent at the cell wall [Feraru 2011]. This experiment indirectly showed that there are some proteins or protein complexes anchored simultaneously at the extracellular matrix and the plasma membrane and that the connection is so strong that is able to withstand the osmotic force. These observations also highlighted that polarly localized PIN proteins in comparison to apolar PIP2 where significantly more accumulating at the cell wall [Feraru 2011]. These results are in line with a more comprehensive study (Chapter 4), describing various polar and nonpolar markers showing differential protein accumulation at the cell wall and plasma membrane following plasmolysis. It revealed that all polar markers are to a greater extend connected to the stabilizing extracellular matrix (Chapter 4). This possibly limits the lateral diffusion of proteins within the plasma membrane as suggested by its increase following partial cell wall digestion [Feraru 2011] This additionally favors a role of extracellular matrix role in protein stabilization at the polar domain.

These initial insights established a role for connections between extracellular matrix and plasma membrane in asymmetric protein distribution. However, we are just at the beginning of the way to discover the underlying molecular mechanisms and regulators. From the cell polarity point of view, the cell wall offers a pronounced capacity for specific protein anchoring what may allow the retention of a number of proteins or even differential endo/exocytosis within single polar domain [Chapter 4, Fig1]. Such a scenario would provide plant cells with flexibility in organization and response to external and internal

cues. It provides also a conceptual possibility to integrate osmotic and mechanic stresses exerted eventually on the whole tissue or organ into regulation of polarity via the extracellular matrix as indicated by previous studies [Traas 2010]. Taking into account that plants are sessile organisms and are often exposed to strong abiotic and biotic stresses, such regulations would have pronounced importance for fitness and survival.

Plasma membrane role in polarity:

Current perception of the biological membranes both in animals and plants do not consider them any longer as a bilayers composed of homogenously distributed lipids and proteins [Lingwood 2010]. In plants these plasma membranes are enriched in sterol and sphingolipids, and depleted in unsaturated phospholipids. The compositional and functional heterogenity of plants plasma membrane has been shown by several lines of evidence. Model membranes showed that mixture of lipids normally found in biological bilayers undergoes 'phase separation' leading to the formation of liquid ordered (Lo), domains enriched in sterol and sphingolipids [Silvius 2005]. Self-associating properties between sterols and highly saturated hydrocarbon chains of sphingolipids have been proposed to constitute the main driving force for membrane segregation *in vivo* and the formation of stable membrane domains [Mongrand 2010]. These so called rafts are defined as a small (10–200 nm), heterogenous, highly dynamic domains that compartmentalize cellular processes [Pike 2006]. Initially, membrane rafts were defined as a low-density TritonX-100 insoluble fraction isolated from tobacco (*Nicotiana tabacum*) PM [Peskan 2000]. Further work in tobacco [Mongrad 2004] and *Arabidopsis* [Borner 2005] revealed the particular lipid composition of these detergent insoluble membranes (DIMs), which started to be considered as a biochemical counterpart of membrane rafts in animals [Mongrad 2004]. However, the methodology, usage of low temperatures for DIMs

isolation and protein insolubility in used detergents generated the issue of artifacts [Munro 2003; Brown 2006].

Plant membranes represent a mix of sterols and sphingolipids. The main sterols of most of the plants are sitosterol, campesterol and stigmasterol. The sterol composition, however, varies significantly across plant taxa [Lefebvre 2007; Furt 2007, Laloi 2007]. The major plant sphingolipids are glycosylinositol phosphoceramides (GIPCs), which contain saturated or mono-unsaturated very long chain fatty acids (with 22–26 carbon atoms) [Pata 2010]. The diversity in composition of sterols and lipids occurs not only at the different domains of the cytosolic plasma membrane surface but also between inner and outer leaflets. In animals the apical surface of epithelial cells is enriched with glycosphingolipids (GSLs) [Hill 1999; Fadeel 2009; Lingwood 2010] and the raft type domains are present in both leaflets of the PM [Fadeel 2009]. In plants, there is also a clear asymmetric distribution of phospholipids, free and conjugated sterols, and glucosylceramide in the PM cytosolic compared with the apoplastic leaflet, with molar ratios of 65:35, 30:70 and 30:70, respectively [Tjellstrom 2010].

A major challenge for the future is the development of tools to enable *in vivo* studies of protein and lipids in the membranes. Visualization of proteins anchored to the outer and inner membrane leaflet would attest to the presence and heterogeneity of membrane domains [Mongrand 2010]. The estimated size of the rafts is far below the optical resolution limit, therefore in order to have better insight into structure and localization one has to turn to Electron Microscopy (EM) and superresolution imaging techniques. However, EM enabling visualization of proteins or lipids at a high resolution is usually performed on cell sections and, therefore, it is not suitable for detecting membrane surface heterogeneity. Nonetheless, studies on animal PM, using protocols involving transmission EM and immunogold labelling of lipids and proteins present in DIMs have revealed a

clustering of lipids and proteins in domains 20–70 nm in diameter [Simons 2000; Manes 2003]. Tracking of the membrane compartments at the cell surface can be investigated by 'evanescent waves' based technology such as TIRF (total internal reflection fluorescence) [Groves 2008] or VIAFM (variable incidence angle fluorescence microscopy) [Konopka 2008]. In animals, it has been demonstrated that, molecular interactions within rafts in living cells can be successfully studied using fluorescence correlation spectroscopy (FCS) [Lasserre (2008)] or Stimulated Emission Depletion (STED) [Eggeling (2009); Sahl (2010)]. In plants, semi-quantitative confocal and super resolution microscopy was successfully used revealing that polarly-localized auxin transporters PIN1 and PIN2 are not evenly distributed in the plasma membrane but show distinct heterogeneity, accumulating in the plasma membrane in so-called 'clusters' [Kleine-Vehn 2011]. Subdiffraction resolution STED microscopy confirmed PIN2 accumulation in membrane clusters but did not detect similar subdomains in case of other auxin carrier AUX1 that shows no polar distribution in the same cells. It has been estimated that PIN2-containing membrane clusters vary on average between 100 and 200nm in diameter. It is thus possible that such protein clustering is linked to their reduced lateral diffusion. Live imaging of PIN1 and PIN2 proteins associated with clusters showed that clustered PINs were largely immobile in the time window of at least 10 min. This was confirmed also by variable angle epifluorescence microscopy (VAEM) observing PIN2 proteins at the lateral cell side [Kleine-Vehn 2011].

Plasma membrane heterogeneity within polar domains, between the different domains or in different cell types is poorly characterized. However so far there are multiple observations confirming that in plants polar localization of proteins indeed depends on the composition of the plasma membrane. It has been shown that defect in sterol biosynthesis leads to PIN1 and PIN3 auxin efflux carriers mis-localization. A mutation in the *STEROL*

METHYLTRANSFERASE1 (*SMT1*, orthologue of yeast *erg6*) gene resulted in altered embryo development, auxin transport and cell polarity [Willemsen 2003]. Another mutant, *cpi1-1*, deficient in the cyclopropylsterol isomerase 1 catalyzing a step following SMT1 in the sterol biosynthesis pathway, also shown defect in PIN2 polarity and in particular post-cytokinetic polarity re-establishment[Men 2008].

Besides genetic evidences, also pharmacological approaches support a notion that sterol composition is linked with polar protein localization. Grebe et al. observed that PIN2 recycling endosomes co-localizes with sterols sharing the same BFA sensitive endocytic pathway [Grebe 2003]. Importantly, treatment with filipin, the sterols biding compound, affects endocytosis, which is of great importance for asymmetric protein distribution [Kleine-Vehn 2006]. These results further support the concept that polarity of PINs, and others polarly localized proteins, at least to some extend partially depends on sterols. It was also shown that, the non-polarly localized auxin carrier ATP-binding cassette transporter B19/P-glycoprotein 19 (ABCB19/PGP19), is enriched in DIMs [Titapiwatanakun 2009] and treatment with sterol chelator methyl-b-cyclodextrin (MCD) releases ABCB19 from membranes, suggesting that phytosterols play a crucial role in this association [Titapiwatanakun 2009]. Overall these data suggest that plant 'rafts' may have a role in maintaining a constant sterol-related, lateral heterogeneity of the PM and in consequence in processes of growth and development [Mongrand 2010].

Conclusions:

Asymmetric distribution of the proteins is fundamental to many aspects of cell and developmental biology in both unicellular and multicellular organisms. In animals the coordinated actions of well conserved Crumbs, Scribble and PAR complexes initiate the formation and ensure maintenance of cell polarity [Wells 2006; Lu and Bilder 2005; Chen

2010]. Apical and basolateral domains are not only controlled by molecular determinants but also a physical barriers tight junctions [Giepmans, Ijzendoorn 2009]. In plants the molecular polarity regulators remain largely unknown. The polarity modules of animals are absent [Geldner 2009], furthermore a tight junction like structure are present but only in endodermal cells and the composition and orientation of this structure is different [Roppolo 2011]. Plant cells possess at least four polar domains apical, basal, outer lateral and inner lateral [Grebe 2010]. Among them the best characterized polar domains are predominantly apical and basal, defined by polar localization of phytohormone auxin carriers PIN1 and PIN2. PIN proteins determine the intracellular transport, direction and rate of auxin flow within tissues and thus can modulate different aspects of auxin distribution-mediated development, including gravitropism, phototropism, embryogenesis, organogenesis, vascular tissue formation and regeneration as well as others [Petrášek 2006; Wiśniewska 2006; Vanneste and Friml, 2009].

At the molecular level, polar PIN targeting depends on cell type- and PIN sequence-specific factors [Wiśniewska 2006] and undergoes a constitutive clathrin-dependent cycling [Dhonukshe 2007]. Interestingly, in a positive feedback mechanism auxin can inhibit clathrin-dependent endocytosis, contributing to self-organinzing auxin-mediated tissue polarization [Sachs 1981; Scarpella 2006; Wabnik 2010]. Regulation of cellular polarity is not only involved in auxin-transport-mediated development, but also in nutrient uptake, the exchange of compounds between roots and the soil as well as in the interaction with pathogens, as suggested by strict outer lateral and inner lateral localization of components of these processes [Miwa 2007; Alassimone 2010; Łangowski 2010; Takano et al, 2010]. The complexity of polar trafficking pathways in plants raises the question of what are the mechanisms for maintaining the asymmetric distribution of proteins. It seems that there are several levels of regulation: (i) intrinsic cargo-sorting signals, (ii) distinct

intracellular trafficking pathways delivering specifically sorted proteins, (iii) heterogeneity of the plasma membrane determining retention and mobility of the cargo, (iiii) and most likely polarity maintaining role of extracellular matrix. According to putative regulatory levels there are four challenges to take. First, identification of the signaling motives within the polar cargos by random or directed mutagenesis. Second, identification of the trafficking regulators by using more and more valuable tool, chemical genomics which allow to bypass the lethality and partially redundancy issues. Third, visualization of the plant rafts by EM and high resolution microscopy. Fourth, identification of the proteins connecting cell wall and plasma membrane by forward and reverse genetics.

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CHAPTER2

Arabidopsis PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid

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Author's Contribution: ŁŁ performed experiments presented in Figure 1B-D, Figure 3A, SFigure 1A-C

Abstract:

Differential distribution of the plant hormone auxin within tissues mediates a variety of developmental processes. Cellular auxin levels are determined by metabolic processes including synthesis, degradation and (de)conjugation as well as by auxin transport across the plasma membrane. Whereas transport of free auxin such as naturally occurring indole-3-acetic acid (IAA) is well characterised, little is known about the transport of auxin precursors and metabolites. Here we identify a mutation in the *ABCG37* gene of *Arabidopsis* that causes the *polar auxin transport inhibitor sensitive1* (*pis1*) phenotype manifested by hypersensitivity to auxinic compounds. *ABCG37* encodes the pleiotropic drug resistance transporter that transports a range of synthetic auxinic compounds as well as the endogenous auxin precursor indole-3-butyric acid (IBA) but not free IAA. ABCG37 and its homolog ABCG36 act redundantly at outermost root plasma membranes and, unlike established IAA transporters from the PIN and ABCB families, they transport IBA out of the cells. Our findings explore possible novel modes of regulating auxin homeostasis and plant development by means of directional transport of the auxin precursor IBA and presumably also other auxin metabolites.

Introduction:

Plants have evolved outstanding capacities to adapt their metabolism and development to respond to their environment. Changes in the availability and distribution of endogenous signalling molecules, plant hormones, play important roles in these responses [Santner 2009]. The phytohormone auxin, perceived by TIR1/AFB receptor proteins and interpreted by downstream nuclear signalling pathway, is an important signal that mediates transcriptional, developmental reprogramming [reviewed in Parry, Estelle 2006; Kepinski,

Leyser 2005]. The differential distribution of auxin within tissues is essential for many adaptive responses including embryo and leaf patterning, root and stem elongation, lateral root initiation, and leaf expansion [Vanneste, Friml 2009]. Differential distribution of the major active auxin, IAA, depends on its intercellular transport and metabolic processes that involve biosynthesis by several pathways and release from storage forms including amide- or ester-linked conjugates with amino acids, peptides, and sugars [Woodward, Bartel 2005]. The role of another endogenously occurring auxinic compound IBA is still unclear. It has been proposed to act independently of IAA [Ludwig-Müller 2000, Plant Growth Regulation 32, 219-230] but number of recent genetic findings suggest that IBA functions as an important precursor to IAA that is converted to IAA by peroxisomal fatty acid β-oxidation [Zolman 2007, Zolman 2008]. Besides metabolism, a crucial process controlling auxin levels in tissues is directional, intercellular auxin transport that depends on specialized influx and efflux carriers [Vieten 2007]. IAA transporters include amino acid permeases-like AUXIN RESISTANT1 (AUX1) mediating auxin influx [Bennett 1996, Yang 2006, Swarup 2008], the PIN-FORMED (PIN) efflux carriers [Luschnig 1998, Petrasek 2006, Wiśniewska 2006] and MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (PGP) class of ATP-Binding-Cassette (ABC) auxin transporters [Noh 2001, Geisler 2005, Bandyopadhyay 2007, Mravec 2008]. Despite the demonstrated importance and wealth of knowledge on the transport of IAA, the mechanism and physiological relevance of transport of its precursors and metabolites remains elusive.

Results:

***pis1* mutant is hypersensitive to exogenous IBA**

To understand mechanisms of auxin homeostasis regulation, we analyzed one of early characterized mutants *polar auxin transport inhibitor sensitive1* (*pis1*) of *Arabidopsis thaliana*, which is hypersensitive to different auxinic (and/or auxin transport interfering) compounds but not to the active, natural auxin IAA [Fujita 1997]. *pis1* mutant roots show strongly enhanced sensitivity to auxinic compounds including synthetic auxins (2,4-D, 2-NOA) and inhibitors of auxin transport (1-NOA, NPA, PBA, TIBA) (Fujita 1997, Fig. 1*A*). When naturally occurring auxins were tested, *pis1* showed normal sensitivity to IAA or PAA, but increased sensitivity to IBA (Fig. 1*A* and S1*A*). To test whether the increased *pis1* sensitivity to auxins is also reflected at the level of auxin signalling, we introduced *DR5rev::GFP* auxin response reporter [Ulmasov 1997, Benkova 2003] into *pis1* plants. Whereas no obvious changes in *DR5* activity were observed on control medium (Fig 1*B*), application of 2,4-D, NPA, or IBA, but not IAA, led to a broad activation of *DR5* expression in *pis1* roots at concentrations markedly lower than in wild-type seedlings (Figs. 1 *C* and *D*, S1 *B* and *C*). Thus, *DR5*-monitored auxin signalling in *pis1* shows increased sensitivity to auxinic compounds similarly to other phenotypic aspects.

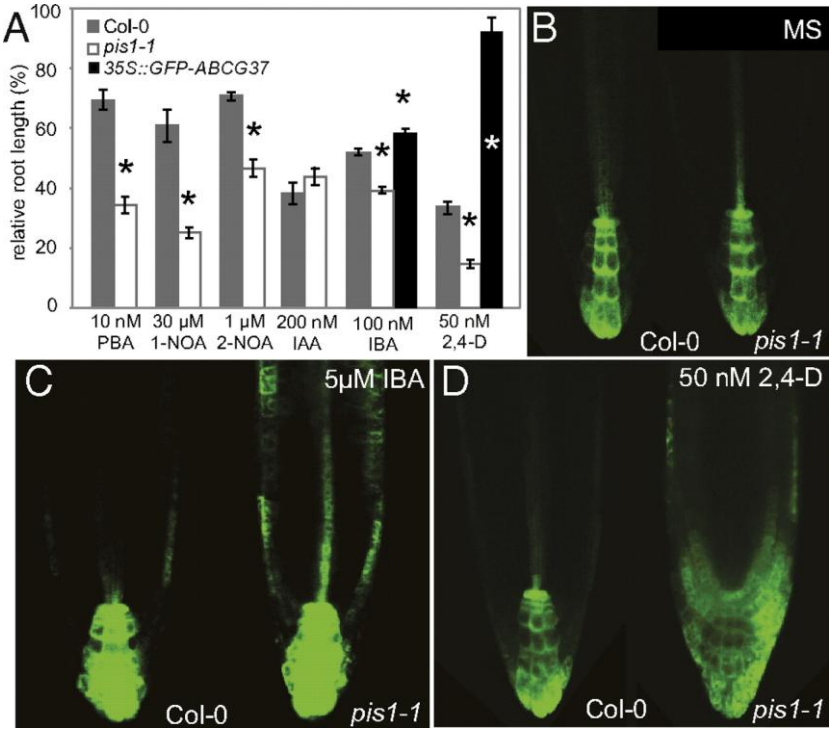


Figure 1. Loss of function *pis1* mutant is hypersensitive to auxinic compounds including natural auxin IBA.

(*A*) *abcg37* (*pis1-1* allele) root growth is hypersensitive to different auxinic compounds (PBA, 1-NOA, 2-NOA, 2,4-D) and the natural auxin precursor IBA but not to active auxin IAA; overexpression of GFP-ABCG37 in *pis1-1* background confers resistance to IBA and 2,4-D (* different from Col-0 control, $P < 0.01$ by ANOVA). (*B*) *DR5::GFP* in the *pis1-1* mutant does not show any detectable difference compared to the wild-type on the control medium. (*C, D*) Hypersensitivity of *pis1-1* to auxinic compounds leads to increased activity of *DR5rev::GFP* auxin response reporter at suboptimal concentrations of IBA (*C*) and 2,4-D (*D*).

PIS1 codes for polarly localized ABCG37 ATP-binding cassette transporter

We mapped the *pis1* mutation using 2800 chromosomes to an 80-kb region on the lower arm of chromosome 3. Sequencing candidate genes revealed a mutation that leads to altered splicing and deletion of 9 amino acids in the gene coding for the previously characterised protein ABCG37/PDR9 [Ito 2006, Strader 2008], a member of the G-subgroup of ATP-binding cassette (ABC) transporters (Verrier 2008, Fig. 2*A*). The altered splicing was confirmed by the sequencing of the ABCG37 cDNA from the *pis1-1*

seedlings (Fig. 2*A*). Allelic complementation analyses of *pis1-1* with the *abcg37* T-DNA insertion mutant (*pdr9-2*, 22) confirmed that the auxin hypersensitivity of *pis1* results from loss of *ABCG37* function (Fig. 2*C*). Moreover, *ABCG37* overexpression in *35S::GFP-ABCG37* lines complemented the *pis1-1* mutation and conferred increased resistance of roots to IBA and 2,4-D (Fig. 1*A*). These and previous [Strader 2008, Verrier 2008] findings on changed auxin sensitivity in loss- and gain-of-function *abcg37* alleles suggest a role of ABCG37 as exporter for auxinic compounds, but this function has not been so far demonstrated directly.

We localized ABCG37/PDR9/PIS1 *in planta* using polyclonal anti-ABCG37 antibodies [Ito 2006] and detected the ABCG37 signal exclusively at the outermost sides of lateral root cap and epidermal cells of the wild-type but not in *pis1-1* (Fig 2*B*, inset) or *pdr9-2* (not shown) root tips. In the *abcg37* gain of function allele *pdr9-1* [Ito 2006], the ABCG37 localization pattern was normal as in the wild-type (Fig. S2*C*). We confirmed this outer polar localization by using GFP-ABCG37 (25, Fig. S2*A* and *B*).

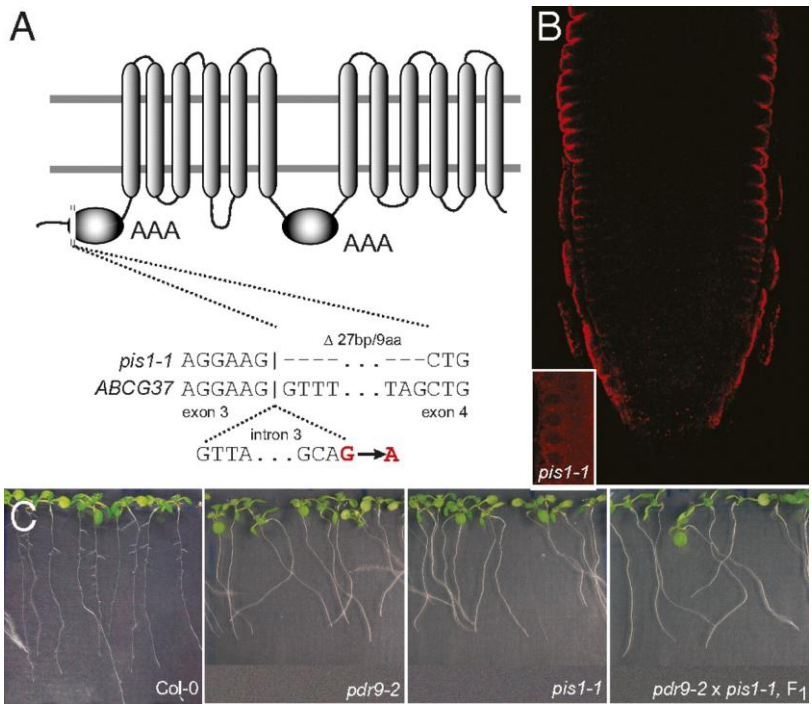


Figure 2. *pis1* mutant carries mutation in the *ABCG37* gene for ATP-binding cassette transporter.

(*A*) The G to A substitution in *pis1-1* affects *ABCG37* splicing, resulting in a 9 amino acid deletion in the first ATP-binding AAA domain. (*B*) ABCG37 is expressed in the epidermis and shows outer polar plasma membrane localization (immunostaining of a primary root tip with anti-ABCG37, inset: absence of the signal in the *pis1-1* mutant). (*C*) *pis1-1* fails to complement the *pdr9-2* mutant allele of *ABCG37* (seedlings germinated on 200 nM NPA, note oversensitivity to NPA manifested by reduced root elongation, lateral root formation and gravitropism).

ABCG36 and ABCG37 act redundantly

Notably, the ABCG37 transporter shows almost identical localization pattern as the homologous ABCG36/PDR8/PEN3 transporter (Fig. 3*A*), which functions in pathogen responses [Stein 2006], cadmium transport [Kim 2007], and also in regulation of IBA sensitivity and IAA homeostasis [Strader 2009]. To uncover possible common roles of these transporters, we generated a double mutant lacking function of both ABCG36 and ABCG37. Root growth assays showed increased sensitivity to IBA of both single mutants and even stronger hypersensitivity of the double mutant (Fig. 3*B*). Nonetheless, the specificity of ABCG36 and ABCG37 action to different compounds does not overlap completely, in particular for synthetic compounds. For example, *abcg37* (Fig. 1*A*) but not *abcg36* [Strader 2008] conveys increased sensitivity to the synthetic auxin 2,4-D, but both act redundantly on its analogue with a longer side chain, 2,4-DB (Fig. S3*D*). Furthermore, these ABC transporters are important for normal development, including root hair elongation (Fig. S3 *A* and *B*), and cotyledon expansion (Fig. S3*C*). Not all aspects of development show similar genetic interactions between *abcg36* and *abcg37*, however. Whereas the double mutant is more sensitive to IBA than either parent in root elongation assays (Fig. 3*B*), the double mutant does not show additive defects in root hair growth (Fig. S3*B*), and shows antagonistic action in cotyledon expansion (Fig. S3*C*). In addition, the

pdr9-1 gain-of-function mutant [Stein 2006] shows opposite phenotypes in root hair growth as compared to the loss-of-function mutant (Fig. S3 *B* and *C*). Altogether, these data suggest that ABCG36 and ABCG37, despite having not completely overlapping properties and showing complex contributions in different tissues, redundantly act on IBA sensitivity and multiple aspects of primary root development.

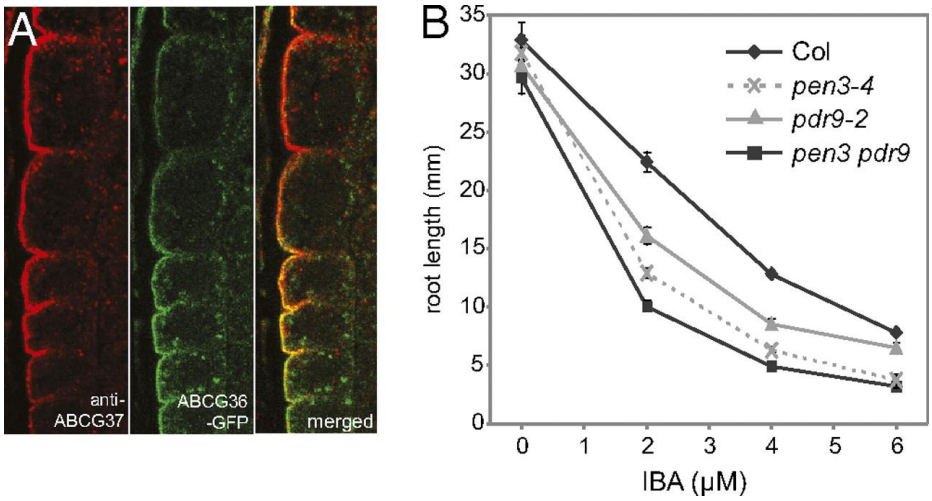


Figure 3. Localization and functional overlap of ABCG37 and ABCG36.

(*A*) ABCG37 co-localizes with ABCG36-GFP in immunolocalization experiments. (*B*) The *abcg36 abcg37* (*pen3-4 pdr9-2*) double mutant shows enhanced sensitivity to IBA compared to either single mutant as manifested by inhibition of root growth (sensitivity of each line was significantly different from others at 2 µM and 4 µM IBA, $P < 0.05$ by ANOVA).

ABGC36 and *ABCG37* regulate IBA accumulation *in planta*

To address the function of ABCG36 and ABCG37 in regulating IBA homeostasis more directly in the place where their localization overlaps, we compared [^{3}H]-IAA and [^{3}H]-IBA accumulation in root tips excised from *abcg36* and *abcg37* single and double mutants. As reported previously [Strader 2008, 2009], *abcg36* and *abcg37* root tips displayed wild-type accumulation of [^{3}H]-IAA but hyper-accumulated [^{3}H]-IBA in this assay (Fig. 4*A*). Importantly, root tips of *abcg36 abcg37* double mutants accumulated even

more [^{3}H]-IBA than single mutants (Fig. 4*A*), consistent with the enhanced sensitivity of the double mutant to IBA in the root elongation assay (Fig. 3*B* and *C*). These results from root were corroborated by transport assays using protoplasts derived from *pis1* mutant leaves. *pis1* protoplasts exported significantly less [^{3}H]-IBA, [^{3}H]-2,4-D and [^{3}H]-NPA than wild type protoplasts, but showed unchanged [^{3}H]-IAA export (Figs. 4*B* and S4*A*). The activity of ABCG37 in leaves protoplasts is in line with altered cotyledon area in various *abcg37* mutants (see Fig. S3*C*), however, it remains unclear what would be the exact physiological role and relevant substrates for the ABCG37-mediated transport in the aerial tissues.

These results demonstrate that ABCG37 acts redundantly with ABCG36 in regulating IBA but not IAA accumulation, presumably acting as exporters of IBA (and other synthetic auxinic compounds) from cells.

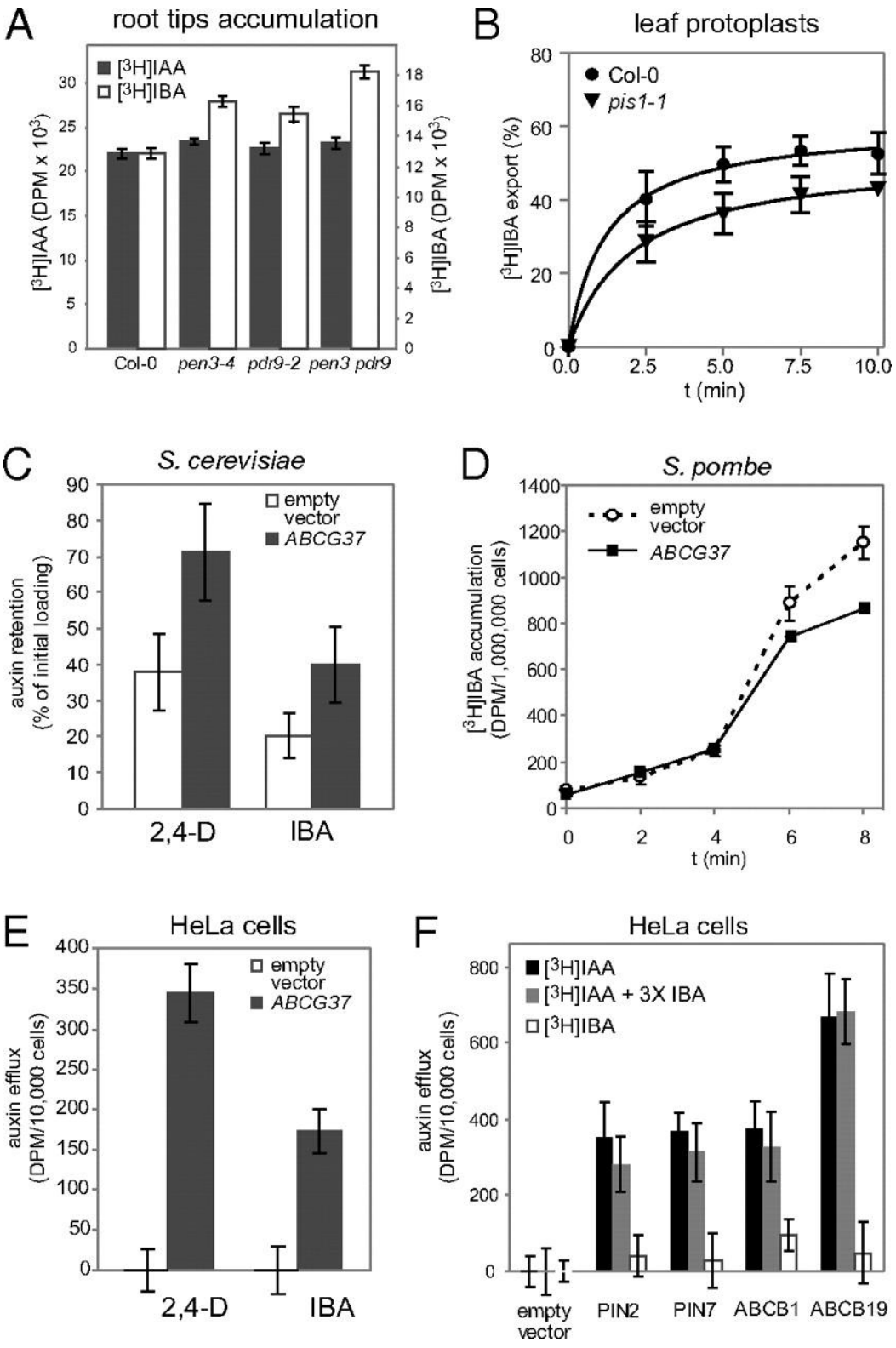


Figure 4. ABCG37 transports IBA and other auxinic compounds.

(*A*) The absence of both ABCG36 and ABCG37 leads to increased [^{3}H]-IBA accumulation ($P < 0.05$ by ANOVA) in root tips, but does not affect [^{3}H]-IAA accumulation. (*B*) *abcg37* (*pis1-1*) leaf mesophyll protoplasts show significantly lower export of [^{3}H]-IBA as compared to the wild-type ($P < 0.05$ by ANOVA). (*C*) Expression of *ABCG37* in *S.*

cerevisiae leads to ABCG37 accumulation in the endoplasmic reticulum and increased retention of [^{3}H]-2,4-D and [^{3}H]-IBA (significantly different from the vector control, Student's *t*-test, $P < 0.05$). (*D*) Expression of *ABCG37* in *S. pombe* cells results in a decreased [^{3}H]-IBA accumulation, significant after 6 minutes ($P < 0.05$ by ANOVA). [^{3}H]-IBA concentration was 250 µM.

(*E*) ABCG37 expression in HeLa cells confers active export of [^{3}H]-2,4-D and [^{3}H]-IBA compared to the empty vector ($P < 0.05$ by ANOVA). (*F*) When expressed in HeLa cells, PIN2, PIN7, ABCB1 and ABCB19 show a clear [^{3}H]-IAA transport ($P < 0.005$ by ANOVA), but no significant [^{3}H]-IBA transport or IBA competition with [^{3}H]-IAA transport was observed. Auxin concentrations were 60 nM [^{3}H]-IAA, 60 nM [^{3}H]-IBA and 180 nM unlabelled IBA (3X IBA). Values shown are means from three replicate experiments.

ABCG37 transports IBA in heterologous systems

To directly test the ability of ABCG37 to export IBA and synthetic auxins, we examined transport activity of ABCG37 expressed in heterologous systems. Expression of ABCG37 in the budding yeast *Saccharomyces cerevisiae*, where it localizes to the endoplasmic reticulum (Fig. S4*B*), led to increased retention of [^{3}H]-2,4-D and [^{3}H]-IBA (Fig. 4*C*), suggesting transport activity of ABCG37 for IBA and other auxinic compounds.

Because the non-plasma membrane localization in *S. cerevisiae* risks uncertain interpretations [Yang 2009], we expressed ABCG37 in a recently established *Schizosaccharomyces pombe* transport system [Yang 2009], where it was localized to the plasma membrane (Fig S4*C*). No significant change in [^{3}H]-IAA transport was found in cells expressing ABCG37, even at concentrations 5-times higher than previously shown for the PIN and ABCB auxin exporters (Fig 4*D*, Yang 2009). In assays with lower [^{3}H]-IBA concentrations, no difference in net accumulation was seen in cell expressing ABCG37 compared to controls in the time interval analyzed (Fig. S4*E*). However, [^{3}H]-IBA saturation of the system resulted in a significant decrease in net accumulation in cells expressing ABCG37 (Fig 4*D*). More rapid diffusive uptake of [^{3}H]-IBA was observed in *S.*

pombe cells compared to [^{3}H]-IAA (not shown), which explains a lag of activity before a difference could be detected (Fig. 4*D*).

We also examined the ability of ABCG37 to transport auxinic compounds in mammalian HeLa cells, which do not contain endogenous ABCG-type proteins [Verrier 2008]. ABCG37 conferred significant export of [^{3}H]-2,4-D and [^{3}H]-IBA (Fig. 4*E*). As reported previously for other ABC-type transporters and PIN proteins [Geisler 2005, Petrasek 2006], ABCG37 showed decreased substrate specificity when expressed in heterologous systems and was able to transport other weak organic acids, including IAA (Fig. S4*F*). Nonetheless, the unchanged sensitivity to IAA of *abcg37* loss- and gain-of-function mutants and lack of transport activity for IAA in root and protoplast assays strongly suggest that IAA is not an endogenous substrate of ABCG37. We also tested the IBA transport activity of the well established IAA transporters PIN1, PIN7 [Petrasek 2006], ABCB1 and ABCB19 [Geisler 2005]. For those proteins, we did not detect any [^{3}H]-IBA transport activity (Fig. 4*F*), concluding that IBA and IAA utilize different efflux transporters. In summary, the data from root, protoplast, and heterologous systems directly demonstrate that ABCG37 acts as an exporter for synthetic auxinic compounds with a broad substrate specificity, which transports the endogenous auxin precursor IBA but presumably not IAA.

ABCG37 function influences auxin transport and homeostasis in the root tip

Next we tested the relevance of ABCG37 transport function to intercellular auxin transport in the root tip. We applied [^{3}H]-IAA, [^{3}H]-2,4-D and [^{3}H]-IBA to the root columella cells of intact roots of wild type and *abcg37* gain- and loss-of-function seedlings (*pdr9-1* and *pdr9-2*, respectively). Consistent with an export function for ABCG37, the

whole root uptake assays (subsequently excised 400 µm root tip segment) showed that uptake of IBA and probably 2,4-D, but not IAA, decreased in the *pdr9-1* gain of function mutant and increased in the *pdr9-2* loss of function mutant (Fig. 5*B*).

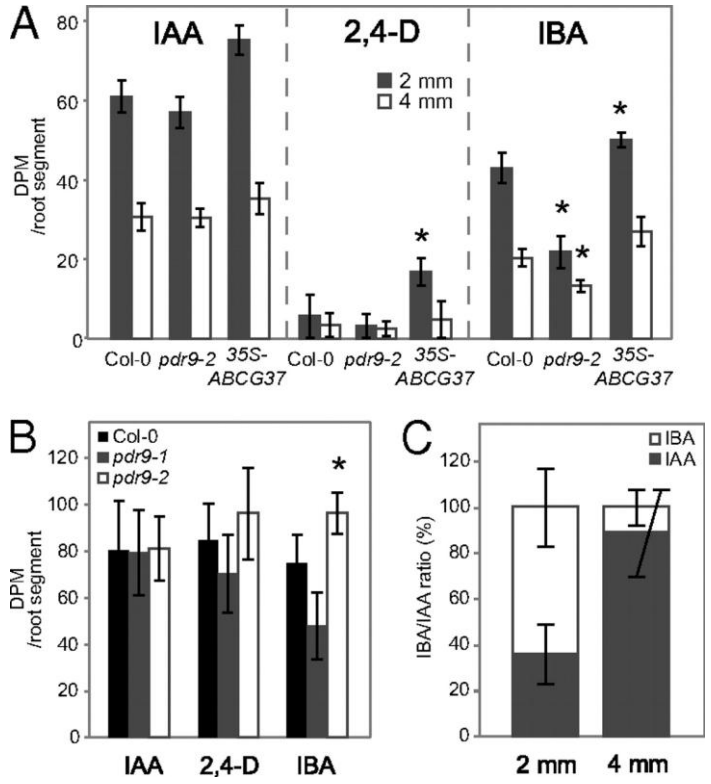


Figure 5. ABCG37 is involved in regulation of auxin homeostasis in the root tip.

(*A*) Basipetal transport of columella-applied auxins: *GFP-ABCG37* overexpression results in an increase in apparent diffusive movement of [^{3}H]-IBA and its non-polarly transported analogue [^{3}H]-2,4-D into the 2 mm segment adjoining the region of application, while loss of ABCG37 function results in decreased basipetal movement of the signal derived from [^{3}H]-IBA application, indicating more specific exclusion of IBA (*$P < 0.05$ by ANOVA). (*B*) Uptake of columella-applied auxins: In a replicate assay, gain (*pdr9-1*) or loss (*pdr9-2*) of ABCG37 function leads to reduced or increased, respectively, uptake of [^{3}H]-2,4-D and [^{3}H]-IBA, but not [^{3}H]-IAA (* significantly different from Col-0, $P < 0.05$ by ANOVA). (*C*) HPLC determination of radiolabelled IAA and IBA obtained from serial sections (0.4-2.4 mm and 2.4 - 4.4 mm from the root apex) 2 hrs after application of 100 fmol [^{3}H]-IBA to root columella cells (ratio in 2 mm section significantly different from 4 mm section, $P < 0.001$ by ANOVA). The results indicate that [^{3}H]-IBA is converted to [^{3}H]-IAA.

We also tested basipetal auxin distribution using a discontinuous media microscale assay [Peer 2007]. Whereas there were no significant changes in transport of [^{3}H]-IAA, the *abcg37* loss-of-function mutant roots showed less transport capacity for [^{3}H]-IBA, and the GFP-ABCG37 overexpressor showed more transport capacity for [^{3}H]-2,4-D and [^{3}H]-IBA (Fig. 5*A*).

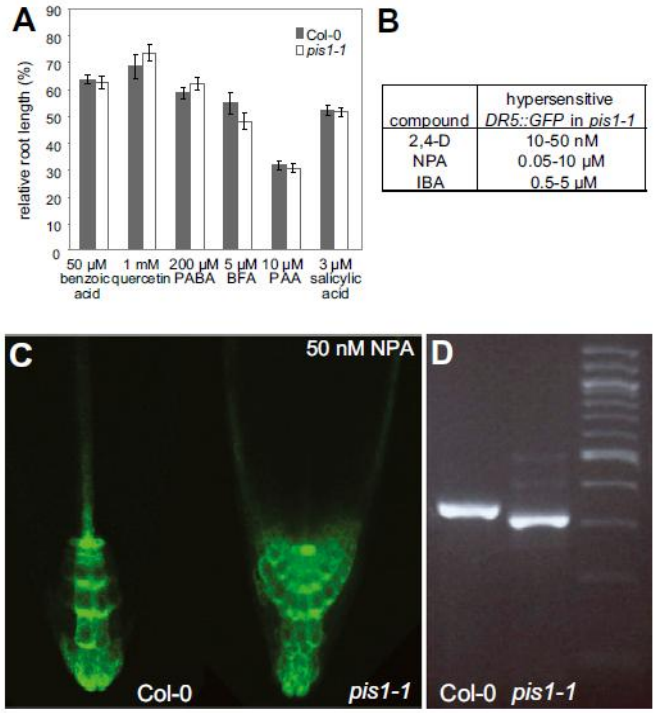
Because IBA is proposed to be an IAA precursor [Woodward 2005], we tested whether IBA is converted to IAA as it moves from the root apex. HPLC analysis of auxins extracted from root segments 2 hours after IBA application on the columella cells revealed that most of the [^{3}H]-IBA is converted into the [^{3}H]-IAA by the time it reaches the region 2.4 - 4 mm above the root apex (Fig. 5*C*). We conclude that ABCG37 regulates auxin distribution and homeostasis in roots by excluding IBA from the root apex, but does not act directly in basipetal transport. Given the rapid conversion of IBA to IAA in the root tip, we hypothesize that ABCG37 might be an additional regulator of auxin homeostasis there.

Discussion:

Differential distribution of the plant hormone auxin within tissues mediates large variety of developmental processes in plants [Santner 2009, Vanneste 2009]. Here we show that in addition to local biosynthesis [Stepanova 2008, Tao 2008, Cheng 2007], subcellular compartmentalization [Mravec 2009], and cell-to-cell transport [Vanneste 2009] of active IAA, auxin distribution can also be influenced by directional transport of the IAA precursor IBA across the plasma membrane. The established auxin exporters [Geisler 2005, Petrasek 2006] do not seem to use IBA as substrate. Physiological data and transport assays from the heterologous systems establish the G-class ATP-binding cassette protein ABCG37 as exporter for IBA. ABCG37 shows a broad substrate specificity for

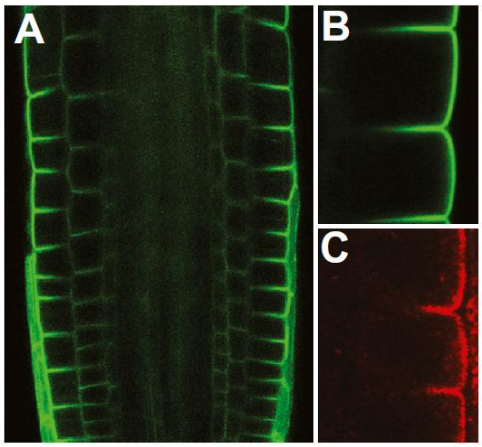
various auxinic compounds including synthetic auxins and auxin transport inhibitors but not the endogenous auxin IAA. It is possible that ABCG37 also transports other auxin metabolites, but this remains to be determined. Given the typical broad substrate specificities of ABCG transporters (Verrier 2008 and references herein), it is also possible that ABCG37 plays a role in transport of other, auxin-unrelated molecules. ABCG37 acts redundantly with ABCG36 in mediating root auxin homeostasis and development. Both proteins show remarkable polar localization at the outermost side of root cells [Langowski 2009, Strader 2009] that implies IBA transport from the root into the surrounding environment. Notably, some microorganisms, including plant symbionts, produce IBA [Martinez-Morales 2003, Badenoch-Jones 1982], raising the intriguing possibility that the ABCG37-dependent transport of IBA, and/or structurally similar compounds, mediates interactions between the root and complex soil microflora.

Supplementary Figure Legends



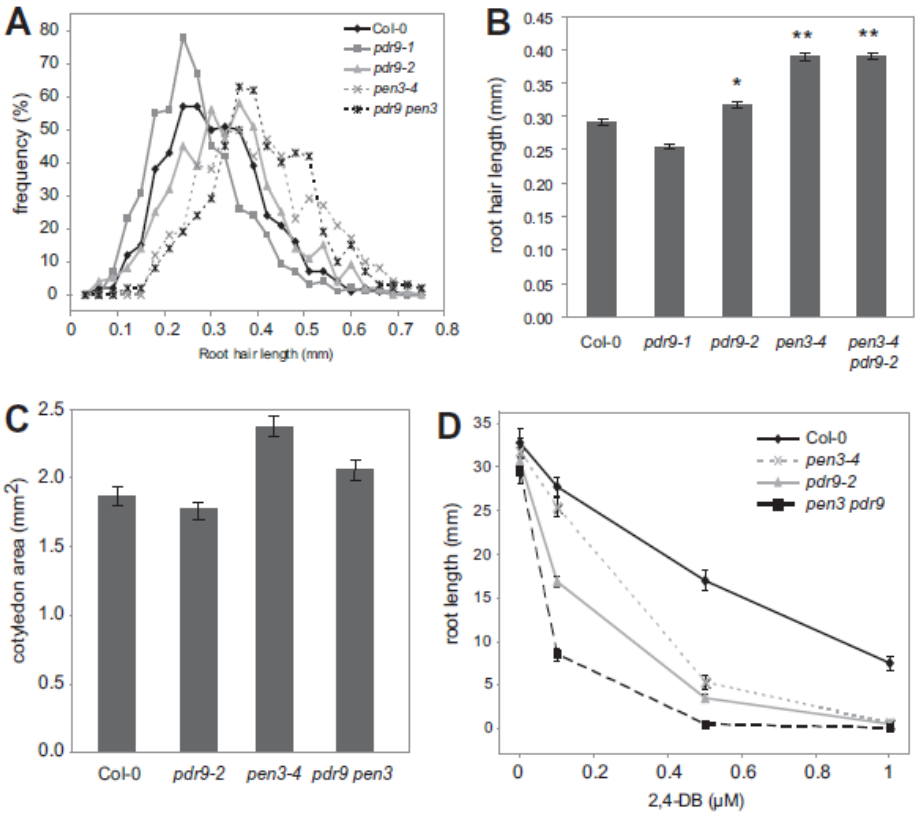
Supplementary Figure 1. Additional analyses of *pis1-1* allele of *ABCG37*.

(*A*) Normal sensitivity of *abcg37 (pis1-1)* to various phytohormone-related substances and compounds structurally similar to auxins ($P > 0.05$ by ANOVA). (*B*, *C*) At given detection limit, the *DR5::GFP* response in *abcg37* (*pis1-1*) is more sensitive to the compound concentration listed than in the wild-type (*B*), a representative image is shown also for NPA (*C*). (*D*) *pis1-1* mutation leads to an aberrant splicing of *ABCG37*. RT-PCR gel image shows the main fragment sequenced, as well as remaining minor transcripts.



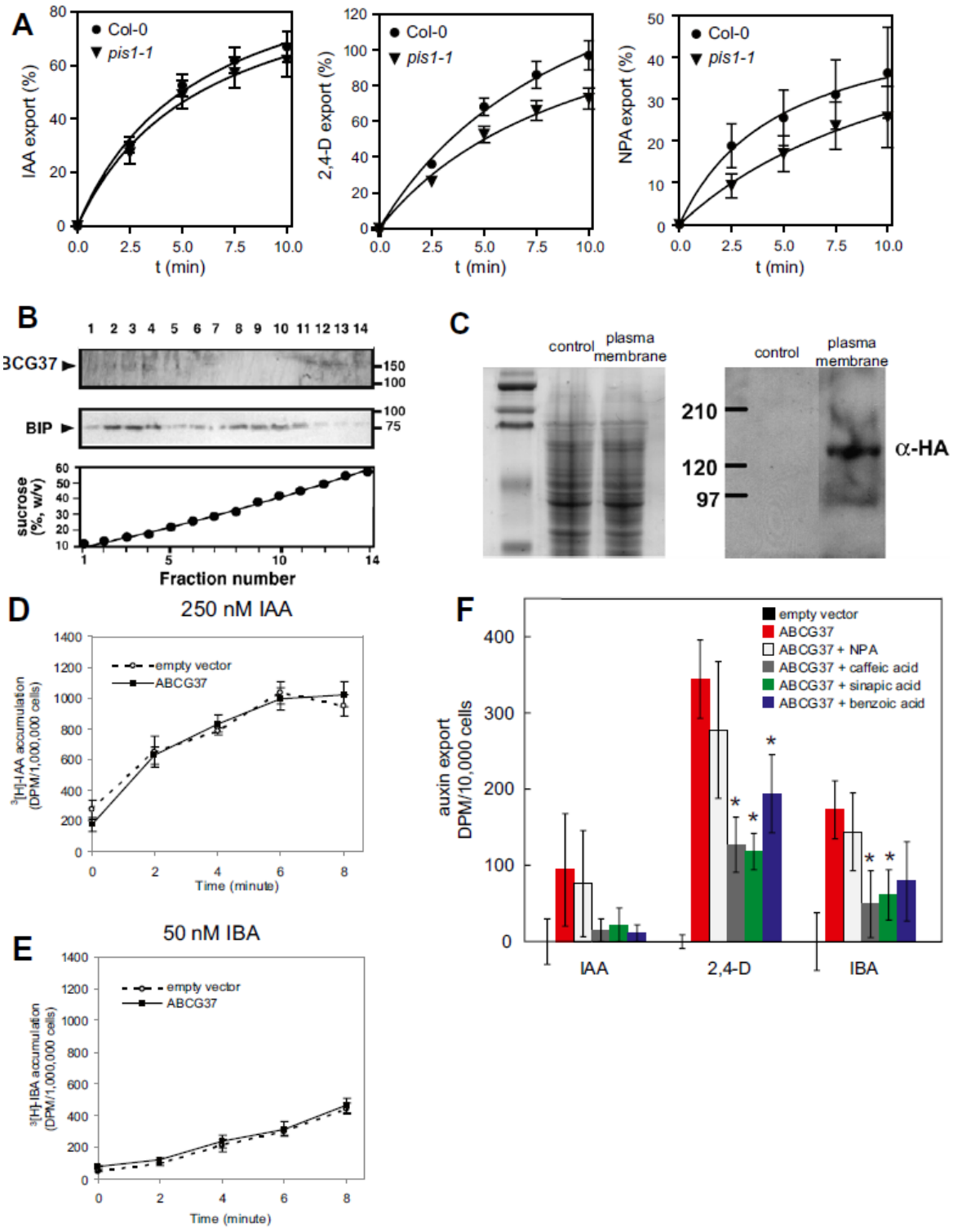
Supplementary Figure 2. ABCG37 is polarly localized at the outer side of root cells.

(*A*) GFP-ABCG37 shows similar polarly localized pattern as observed by anti-ABCG37 immunolocalization. (*B*) magnified view of epidermal cell files. (*C*) ABCG37 is normally localized in *abcg37/pdr9-1* gain of function mutant in immunolocalization experiments.



Supplementary Figure 3. Additional analyses of *abcg37* and *abcg36* phenotypes

(*A, B*) Loss of function (*pdr9-2* and *pen3-4*) or gain of function (*pdr9-1*) in *ABCG37* and *ABCG36* genes increases or decreases root hair frequency (*A*) (significantly different for each line, $P < 0.05$ by χ^2-test) (*B*) and root hair length (* different from *pdr9-1*, ** different from Col-0, $P < 0.05$ by ANOVA). (*C*) Loss of *ABCG37* (*pdr9-2*) partially suppresses the increased cotyledon area conferred by loss of *ABCG36* (*pen3-4*) (Col-0 was different from *pen3-4*; *pdr9-2* was different from *pen3-4* and *pen3-4* and the double mutant, $P < 0.05$ by ANOVA). (*D*) Double *abcg36 abcg37* (*pen3-4 pdr9-2*) roots show enhanced sensitivity to 2,4-DB compared to either single mutant (single mutants were significantly different from the Col-0 control and the double mutant a 0.5 µM concentration point, $P < 0.05$ by ANOVA).



Supplementary Figure 4. Characterisation of ABCG37, ABCB1, ABCB19 and PIN transport.

(*A*) *abcg37* (*pis1-1*) leaf mesophyll protoplasts show significantly lower export of [^{3}H]-2,4-D and [^{3}H]-NPA as compared to the wild-type ($P < 0.05$ by ANOVA at 7.5 min time point), whereas [^{3}H]-IAA export shows no significant difference between the two lines. (*B*) Immunoblotting of sucrose gradient fractions of yeast membrane extracts prepared from yeast expressing ABCG37 demonstrates that ABCG37 is enriched in comparable fractions

as the marker BIP, indicating the ER localization of ABCG37 in yeast. (*C*) Two phase partitioning demonstrates that *ABCG37* is localized on the plasma membrane in *S. pombe* (left: Coomassie blue stained SDS-PAGE gel, right: corresponding western blot). (*D*) ABCG37 does not transport IAA, even at its saturating concentrations in *S. pombe* assays. (*E*) ABCG37 shows no significant transport capacity for lower amounts of IBA in *S. pombe* assays. (*F*) ABCG37 shows a broader specificity in HeLa cells assays (* compound significantly competed with auxin tested, $P < 0.05$ by ANOVA).

Materials and Methods

Material and growth conditions

Arabidopsis seedlings were grown under a 16 hours photoperiod, 22/18 °C, on 0.5 x MS medium with sucrose as described [Benkova 2003], unless indicated otherwise. The following mutants, transgenic plants and constructs have been described previously: *pis1-1* [Fujita 1997], *pdr9-2* [Ito 2006], *pen3-4* [Stein 2006], *DR5rev::GFP* [Benkova 2003]. For *35S::GFP-ABCG37*, the *ABCG37* genomic fragment was cloned into a pBluescript-derived pEPA vector [Dhonukshe 2007]. The fusion construct was subcloned into binary pML-BART [Gleave 1992] and transformed into *pis1-1* mutants.

The following chemicals were used: 2-(1-pyrenoyl)benzoic acid (PBA), 1- and 2-naphtoxyacetic acid (1- and 2-NOA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenoxybutyric acid (2,4-DB), indole-3-butyric acid (IBA), benzoic acid, quercetin, *p*-aminobenzoic acid (PABA), phenylacetic acid (PAA), salicylic acid (all Sigma), brefeldin A (Molecular Probes).

Localization analysis, confocal microscopy

DR5rev::GFP signal in 5 days old seedlings in *Arabidopsis* was observed as described [Benkova 2003]. For 2,4-D and NPA experiments, plants were germinated on selected compound. For IBA and IAA *DR5rev::GFP* observations, in order to minimize potential metabolic conversions, seedlings were incubated in the auxin supplemented liquid medium for 4 hours.

Immunolocalizations in 5 day old seedlings were done as described [Sauer 2006] with anti-ABCG37 [Ito 2006] (1:500) and CY3-conjugated anti-rabbit (1: 600, Dianova)

antibodies. For confocal laser scanning microscopy, a Leica TCS SP2 equipment was used. Images were processed in Adobe Photoshop.

Mutation characterization and double mutant isolation

The predicted *pis1-1* mutation in the donor splicing site was confirmed by RT-PCR and sequencing of the prevailing misspliced product.

All mutants were in the Col-0 accession. The *pdr9-2* (SALK_050885) mutant [Ito 2006] was crossed to the *pen3-4* (SALK_000578) mutant [Stein 2006]. PCR analysis of F_2 plants was used to identify double mutants. *pdr9-2* was identified as previously described [Strader 2008]. Amplification of *PDR8* with PDR8-1 (GTATCACCCAACTAAATCCTCACG) and PDR8-2 (ATCTGTTACACGGCCAAAGTTAG) yields a 1450-bp product in wild type and no product in *pen3-4*. Amplification with PDR8-1 and LB1-SALK yields an ~450-bp product in *pen3-4* and no product in wild type.

Phenotype analysis

The root growth compound sensitivity of *pis1-1* was tested at 3-8 concentrations on six-days-old seedlings, while root length on control media reached approximately 20 mm (corresponds to 100 %, *pis1-1* root length was not significantly different from wild type [Fujita 1997]). At least 15 seedlings were processed for each concentration and at least three independent experiments were done, giving the same statistically significant results; representative experiments are presented. Equal variances of values were verified by Levene test and Kruskal-Wallis non-parametric test was performed simultaneously with ANOVA. Data were statistically evaluated with NCSS 2007.

For IAA, IBA, and 2,4-DB responsive root elongation assays of double mutants, primary root lengths of seedlings grown for 8 days with the indicated auxin concentration were measured. Seedlings were grown at 22 °C under continuous illumination through yellow long-pass filters to slow indolic compound breakdown [Stasinopoulos 1990].

For cotyledon expansion assays, cotyledons of 7-day-old seedlings grown under

continuous white light at 22 °C were removed and mounted. Cotyledons were imaged using a dissecting microscope and cotyledon area was measured using NIH Image software (http://rsb.info.nih.gov/ij/).

For root hair assays, roots of 5-day-old vertically-grown seedlings grown under continuous white light at 22 °C were imaged using a dissecting microscope and root hair lengths from 4 mm of root adjacent to the root-shoot junction and measured using NIH Image software.

Transport measurements

Auxin accumulation in excised root tips was measured as described [Strader 2009]. Leaf protoplast transport assays were performed as described [Geisler 2005]. Available microarray databases predict a moderate leaf ABCG37 expression [Zimmermann 2004], at similar levels as examined PIN proteins [Geisler 2005].

S. cerevisiae assays were done as described [Geisler 2005] with *ABCG37* cDNA cloned as HA-tagged version into the *Not*I site of the yeast shuttle vector, pNEV [Sauer 1994]. Relative IAA export was calculated from retained radioactivity as follows: (radioactivity in the yeast at t = 10 min.) - (radioactivity in the yeast at t=0) * (100%)/(radioactivity in the yeast at t = 0 min.). *S. pombe* assays were done as described [Yang 2009], where a pTM isolated cDNA fragment of *ABCG37* was subcloned into the pREP41 vector; the results show the accumulation of the radioactivity in cells. Determination of ABCG37 plasma membrane localization was done by two phase partitioning followed by western blot as published [Premsler 2009]. Transport activities in HeLa cells were determined as reported [Geisler 2005, Petrasek 2006]. Net efflux is expressed as DPM/10,000 cells divided by the amount of auxin retained by cells transformed with empty pTM1 vector minus the amount of auxin retained by cells transformed with *ABCG37*. The data presented are averaged data sets from three independent experiments. Student's *t*-tests were run for individual pairwise comparisons and then compared by ANOVA using Newman-Keuls post hoc test, followed for *P* values close to 0.05 by Dunnett's and Tukey tests.

The IBA transport measurements of auxin exporters were conducted as described [Blakeslee 2007], using 60 nM [^{3}H]-IAA (21 Ci.mmol^{-1}), 60 nM [^{3}H]-IBA (18.9 Ci.mmol^{-}

[1], HPLC purified), both from American Radiolabeled Chemicals, and 180 nM unlabeled IBA (Sigma).

Root tip applied auxin transport was measured as described [Geisler 2005, Peer 2007], with following modifications: A 10 nL (root tip uptake) or 6 nL (basipetal root transport) droplet with 1 µM radioactively labelled auxin ([^{3}H]-IAA, 18 Ci.mmol^{-1}; [^{3}H]-2,4-D, 21 Ci.mmol^{-1}; [^{3}H]-IBA, 21 Ci.mmol^{-1}, American Radiolabeled Chemicals) was applied on the third tier of columella cells. After 2 hrs, the root cap was removed and radioactivities of excised 2 mm root tip segments were measured. [^{3}H]-IBA was repurified by HPLC to remove contaminants prior to use.

The HPLC analysis of transported [^{3}H]-IBA signal was accomplished by extraction in methanol and separation in a 10-100% methanol/2% formic acid gradient with radiodetection compared to IAA and IBA standards. The significance was tested by ANOVA followed by Newman-Keuls test.

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CHAPTER3

Trafficking to the outer polar domain defines the root-soil interface

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Author's Contribution: JF initiated the project; JF and ŁŁ designed the experiments; ŁŁ carried out most of the experiments; KR performed experiments presented in Figure 1G-J and Figure 2A; SN performed experiments presented in Figure 2B; JK performed experiments presented in Figure 1L; ŁŁ performed experiments presented in Figure 1A-F, K; Figure 3A-K, Figure 4D-J; Figure S1A-I; Figure S2A-K; Figure S3A-G; Figure S2A-F; ŁŁ associated the figures, ŁŁ and JF discussed the results and wrote the manuscript.

Abstract:

In animals, the interface between organism and environment is constituted by the epithelium, of which the functionality is assured by a selective localization of proteins to the opposite apical and basolateral polar domains [Drubin 2000]. In plants, the exchange of nutrients and signals between root and soil is crucial for their survival, but the cellular mechanisms underlying the epithelium-like function and specific localization of proteins to the root surface have not been identified [Gojon 2009]. Here we analyze the mechanism of polar delivery to the root-soil interface of proteins BOR4, ABCG37, and PEN3 which transport nutrients [Gojon 2009] and plant hormones [not published] or are required for pathogen defense [Parniske 2008], respectively. The simultaneous visualization of these proteins and apical and basal cargos in a single cell demonstrates that outermost cell side represents an additional polar domain in plant cells. Delivery to this outer polar domain is based on the ARF GEF- [Donaldson 2000] and actin-dependent mechanism [Rahman 2007, Kleine-Vehn 2008, Cárdenas 2008, Dhonukshe 2008], and is, in contrast to known basal and apical cargos [Dhonukshe 2008, Tanaka 2009], mediated by the polar secretion. Importantly, the outer polar localization does not require the known molecular components of the apical or basal targeting. Our findings show that outermost cell membrane of roots defines an additional polar domain in plant cells along with a specific, previously uncharacterized polar targeting mechanism that is important for defining the functional, epithelium-like root-soil interface.

Results and Discussion:

The interface between roots and soil plays a central role in the plant's life. Uptake of nutrients [Gojon 2009], export or exclusion of toxic compounds [Verbruggen 2009], interaction with symbionts and pathogens [Parniske 2008] as well as exchange of other signals require a functional interface between roots and surrounding [Drubin 2000]. Recently, several proteins have been reported to reside at the outer lateral side of root epidermis cells that forms the border with the soil environment. These include the transporter for the plant nutrient boron, BOR4 [Miwa 2007], the exporter of the plant hormone precursor indole-3-buryric acid (IBA) PIS1/PDR9/ABCG37 [Ito 2006; unpublished data], and the pathogen defence-related transporter PEN3/PDR8/ABCG36 [Kobae 2006, Stein 2006, Strader 2009]. Nonetheless, almost nothing is known about how this interface between roots and soil is defined or by which mechanism transporters and other regulatory proteins specifically accumulate at this outermost root plasma membrane.

The subcellular distribution of BOR4, PEN3, and ABCG37 was analyzed in the *Arabidopsis* transgenic lines *35S::BOR4-GFP*, *PEN3::PEN3-GFP*, and *35S::GFP-ABCG37* as well as with antisera against ABCG37 [Ito 2006]. BOR4-GFP in *35S::BOR4-GFP* roots showed less strict polar localization; it resided predominantly at the outer membrane of epidermis cells but to some extent also at the apical and basal membranes, particularly, in older, more differentiated cells (Figure 1 *D*; Figure S1 *G*). On the other hand, ABCG37, GFP-ABCG37, and PEN3-GFP showed more strict polar localization at the outer sides of epidermis and root cap cells (Figure 1 *A-C*; Figure S1 *H* and *I*). The colocalization experiments between ABCG37, BOR4-GFP, and PEN3-GFP confirmed that the localization domain of BOR4 was less strictly polar then that of ABCG37 and PEN3 (Figure 1 *E* and *F*; Figure S1 *A-F*). To test, whether the less pronounced polar localization of BOR4-GFP is a result of overexpression and possible saturation of the trafficking

machinery, we compared BOR4-GFP, GFP-ABCG37, and PEN3-GFP signal intensities using the same confocal microscope settings (Figure S1 *G-I*). GFP-ABCG37 in *35S::GFP-ABCG37* showed the strongest signal intensity but less exclusive lateral localization as compared to the endogenous ABCG37 detected by antibodies. On the other hand, the BOR4-GFP in *35S::BOR4-GFP* showed comparable or weaker signal as compared to more strictly localized PEN3-GFP in *PEN3::PEN3-GFP* roots. Thus, it is unlikely that the less pronounced polar localization of BOR4-GFP is the result of overexpression but it rather reflects the less strict targeting of BOR4 as compared to ABCG37 and PEN3.

To examine the occurrence of the outer lateral domain in different cell types, we examined the *35S::GFP-ABCG37* transgenic line expressing ectopically the functional GFP-ABCG37 throughout all the cell types of the root. Similarly to the endogenous protein, GFP-ABCG37 was detected at the outer sides of epidermal cells and also to outer lateral sides of cortex and endodermal cells but, in more interior root cell types, its localization was symmetric (Figure 1 *G* and *H*), even in differentiated stele cells (not shown) suggesting that the outer polar domain is specified in the three most external cell layers but not in the more interior cell types. Notably, transversal and longitudinal optical sections revealed that ABCG37, and PEN3 strictly localized to the cell sides facing the environment (Figure 1 *I*), regardless of the position and apical-basal axis of the cells. For example, in the root cap cells at the very root tip these proteins localized again to the outermost cell sides, which corresponded to the "basal" sides (Figure 1 *J*). Such strictly outer localization would have an obvious functional importance for transporting compounds between the root interior and the environment. In line with this hypothesis, ABCG37, PEN3 and BOR4 show strong plasma membrane localization in root hairs (not shown), that are increasing surface of the interface with soil and are important for nutrient uptake.

In plants, apical and basal polar domains in different cell types are designated by localization of several polar cargos prominent among them transporters for the plant hormone auxin [Swarup 2001, Wiśniewska 2006, Geisler 2006, Petrasek 2006, Boutté 2007]. Epidermal root cells of *Arabidopsis thaliana* possess an apical domain at their upper side that is defined by the localization of the PIN2 auxin efflux carrier and a basal domain marked by PIN1 [Wiśniewska 2006]. The distinct polar localization of BOR4, ABCG37, and PEN3 in the same cells suggest existence of the additional, and largely uncharacterized polar domain at the outer sides of cells on the root surface. To confirm this notion, colocalization experiments were carried out with apical and basal polar cargos, exemplified by the PIN2 and PIN1 auxin transport proteins, respectively [Wiśniewska 2006]. These experiments revealed that PIN1, PIN2, and ABCG37 localized concomitantly to the three different polar domains in the root epidermal cells (Figure 1 *K* and *L*). This observation of three different proteins at three different polar domains in the same cell explicitly demonstrated that the localization of BOR4-GFP and mainly ABCG37, and PEN3-GFP define a third, additional polar domain in plant cells. Given the unique characteristics of this domain that always faces the outer environment, we designated it as "outer" polar domain.

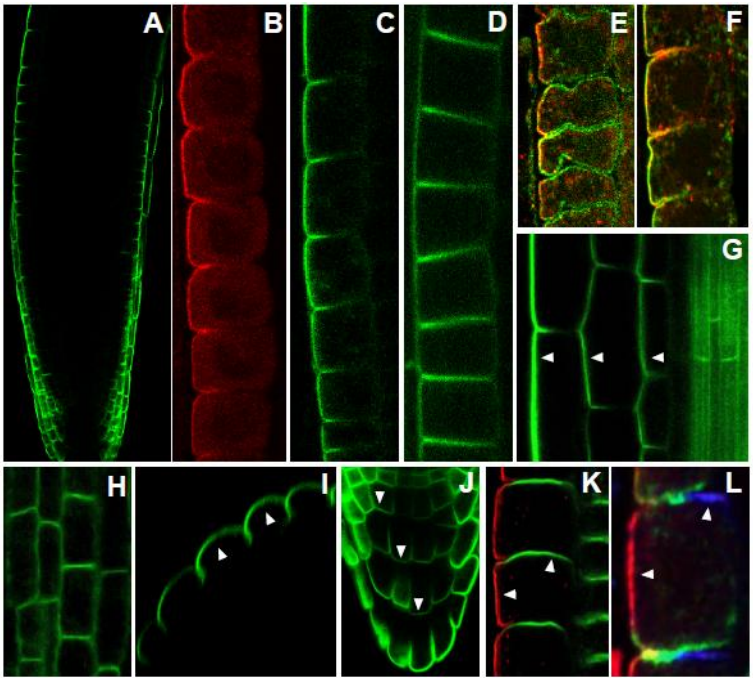

Fig. 1. Novel outer lateral domain.

(*A-D*) Polar localization of PEN3-GFP (*A* and *C*), ABCG37 (*B*), and BOR4-GFP (*D*) at the outer sides of epidermal cells or the *Arabidopsis* root tip. (*E-F*) Co-localisation of BOR4-GFP (green) and ABCG37 (red) (*E*) reveal less strictly polar localization of BOR4 when compared to co-localization of ABCG37 (red) and PEN3-GFP (green) lateral localization (*F*). (*G-H*) Localization of GFP-ABCG37 to the outer polar domain (arrowheads) of epidermal, cortex, and endodermis cells (*G*), but symmetric localization in the stele (*H*). (*I*) Confirmation of GFP-ABCG37 localization to the outer cell sides by transversal optical sections. (*J*) Outer polar domain of GFP-ABCG37 always directed out from the plant body in the root cap. (*K*) ABCG37 (in red) defines the outer polar domain in epidermal cells, additionally to the PIN2-marked apical domain (green). (*L*) Concomitant localization of ABCG37 (red) at the outer, PIN1 (green) at the basal, and PIN2 (blue) at the apical polar domain. Immunostaining with anti-ABCG37 antibodies (*B, E, F, K, L*), life cell imaging (*A,C,D, G-J*). Arrowheads indicate polar localization of proteins.

Polar localization of PIN proteins to the apical and basal cell sides had been shown to be achieved by a non-polar secretion, the subsequent internalization and endocytic recycling-dependent polarization [Dhonukshe 2008, Tanaka 2009]. The Fluorescence Recovery After Photobleaching (FRAP) experiments revealed that after complete bleaching of BOR4-GFP, GFP-ABCG37 or PEN3-GFP the newly synthesized proteins

appeared at the plasma membrane in a polar fashion at the earliest detectable recovery stages (Figure 2 *A* and *B*; Figure S2 *J*), in contrast to the originally non-polar recovery of PIN1-GFP of PIN2-GFP proteins [Dhonukshe 2008b]. This result is a first demonstration of polar secretion in plants and suggests that unlike in case of known apical and basal cargos, the polar localization of outer lateral cargos is established already during polar secretion of the newly synthesized protein.

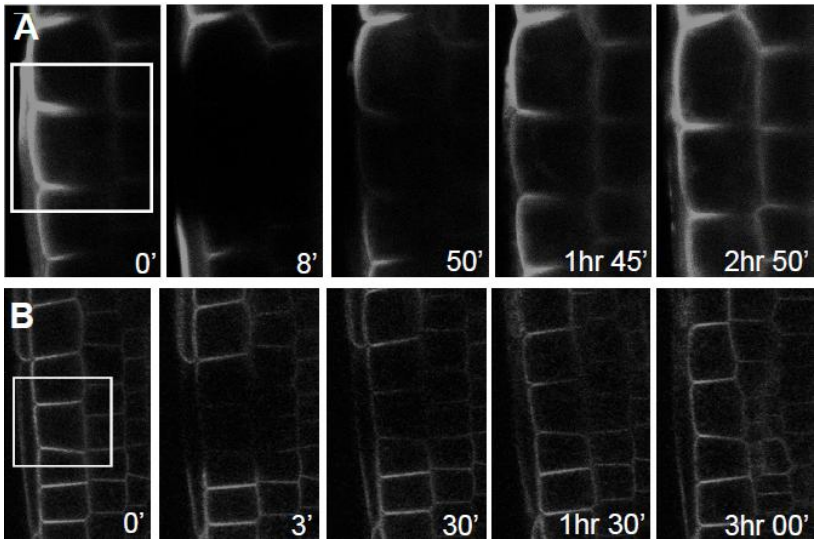

Fig. 2. Secretion to outer lateral domain.

After complete bleaching of the GFP-ABCG37 (*A*) and BOR4-GFP (*B*), both proteins recover directly at the outer polar domain suggesting direct outer polar secretion of newly synthesized proteins. Numbers indicate time points from the bleaching. 0' is a pre-bleach with indicated bleaching area.

The secretion and recycling processes can be studied using the inhibitor of vesicle trafficking the fungal toxin Brefeldin A (BFA) that targets vesicle budding regulators ARF GEFs [Donaldson 2000]. The polar localization of PIN and AUX1 cargos to the basal and apical domains depends on distinct pathways, which are sensitive to BFA. The BFA treatment leads to internalization of PIN1 from the basal plasma membrane and its accumulation in so-called BFA compartments (Figure S2 *D* and *E*) [Geldner 2001, Kleine-

Vehn 2006, Kleine-Vehn 2008]. BOR4-GFP, ABCG37, and PEN3-GFP did not show a comparable sensitivity to BFA and their localization at the outer polar domain remained unaffected after BFA treatment (Figure 3 *A-E* and *H*; Figure S2 *A* and *B*). Nonetheless, these proteins, in particular when overexpressed, showed limited intracellular aggregations. This BFA-induced aggregations were strongly diminished when the *de novo* protein synthesis was inhibited by cycloheximide (Figure 3 *F-I*). Since cycloheximide does not have any detectable effect on endocytosis as tested by uptake of endocytic tracer FM4-64 (Figure S2 *F-I*), this result suggests that limited aggregations after BFA treatment consists of de novo synthesised protein passing the secretory pathway. This notion was further supported by FRAP experiment with cyclohexamide treatment (Figure S2 *J* and *K*). After complete bleaching of PEN3-GFP signal we observed strongly delayed recovery and general signal depletion of the signal from the plasma membrane. Taking into account all the data concerning BFA and CHX effect on BOR4, PEN3 and ABCG37 proteins trafficking, it seems that intracellular agglomerations following BFA treatments consists predominantly from *de novo* synthesized proteins being secreted to the outer polar domain. This contrasts with the behaviour of PIN proteins, where the BFA-induced aggregations persist also in the presence of cycloheximide, reflecting a constitutive endocytic recycling of PIN proteins [Geldner 2001]. This observation suggests that the outer polar cargos are delivered to their polar domain by the BFA-sensitive, ARF GEF-mediated polar secretion, which further highlights the differences in trafficking of the apical and basal cargos versus the outer polar cargos.

In plants, most of the intracellular trafficking, including the targeting of apical and basal cargos, depends predominantly on the actin cytoskeleton, but not directly on the microtubules [Rahman 2007, Kleine-Vehn 2008, Cárdenas 2008, Dhonukshe 2008]. Actin depolymerisation by Latrunculin B (LatB) did not visibly affect the outer lateral

localization of BOR4-GFP (data not shown), GFP-ABCG37, or PEN3-GFP (Figure 3 *J*; Figure S3 *D* and *E*). The outer polar signal remained stable and the limited intracellular aggregations were again mostly related to the secretion of the *de novo* synthesized proteins as demonstrated by their disappearance following cycloheximide treatment (Figure S3 *C*, *F* and *G*). Similarly, the disruption of microtubules by oryzalin did not affect strongly the outer polar localization or trafficking of BOR4-GFP (data not shown), GFP-ABCG37 and PEN3-GFP (Figure 3 K; Figure S3 *A* and *B*). Thus, the actin or tubulin cytoskeleton was seemingly not directly involved in the maintenance of the outer polar localization, but actin seems to be required for the secretion of the *de novo* synthesized outer domain-resident proteins.

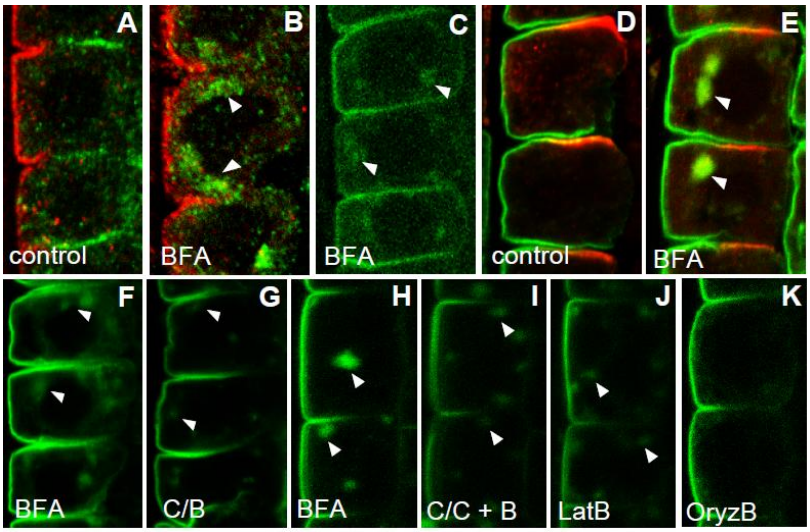

Fig. 3. Involvement of ARF GEF, actin and microtubules in outer lateral targeting.

(*A*-*I*) BFA-sensitive trafficking. BFA treatment (50 µM, 30 min) leads to strong internalization of the basal PIN1-HA (green), but not of laterally localized ABCG37 (red) (*B*) or BOR4-GFP (*C*); untreated control (*A*). When overexpressed under the 35S promoter, the outer localized GFP-ABCG37 shows stronger intracellular agglomerations after BFA treatment, but no signal depletion from the plasma membrane similar to that of the apical PIN2 (red) (*E*); untreated control (*D*). Decrease in intracellular agglomeration of GFP-ABCG37 (*F*) and PEN3-GFP (*H*) after BFA treatment when pre-treated with the protein synthesis inhibitor cycloheximide (50 µM, 30 min) (*G* and *I*), suggesting that not the plasma membrane localization, but the secretion of *de novo* synthesized ABCG37 and

PEN3 is sensitive to BFA. (*J-K*) Actin filaments and microtubules involvement. Depolymerization of microtubules by oryzalin B (50 µM, 3 h) (*K*) or depolymerisation of actin filaments by Latrunculin B (20 µM, 3 h) (*J*) leads to very limited intracellular agglomerations of PEN3-GFP (*C*), however in both cases without effect on its outer polar localization (see also Figure S3 *A-G*). Immunostaining with anti-ABCG37 antibodies (*A, B, D, E, F, G*), life cell imaging (*C, H-K*). Arrowheads indicate intracellular aggregations.

Next, we investigated the molecular mechanism underlying the localization of cargos at the outer polar domain in comparison to the apical and basal targeting. The targeting of basal cargos, such as PIN1, requires action of a specific ARF GEF called GNOM [Geldner 2003, Kleine-Vehn 2006]. Accordingly, in partial loss-of-function *gnom* mutants, the PIN1 and other basally localized proteins show a disrupted polar localization, leading to a partial or complete apicalization [Kleine-Vehn 2008]. Similar analysis revealed that localization of BOR4-GFP and ABCG37 at the outer polar side was not affected in different partial loss-of-function alleles of *gnom* (Figure 4 *B* and *D*). These data show that targeting to the outer polar domain is distinct from the GNOM-dependent basal PIN targeting.

The apical targeting of the AUX1 auxin transport protein does also not depend on GNOM, but on the ER-localized AXR4 protein. In the *axr4* mutant [Hobbie 1995], the otherwise apically localized AUX1 is retained in the ER [Dharmasiri 2006]. In contrast, the BOR4-GFP, ABCG37, and PEN3-GFP localization to the outer polar domain remains entirely unaffected in *axr4* roots (Figure 4 *C* and *E*), highlighting a difference between the outer polar and AUX1 apical targeting. This observation is not surprising because the AXR4 is presumably mediates trafficking specifically of AUX1 [Dharmasiri 2006] and other apical cargos will use an AXR4-independent mechanism. Nonetheless, testing other specific components of apical targeting awaits their identification.

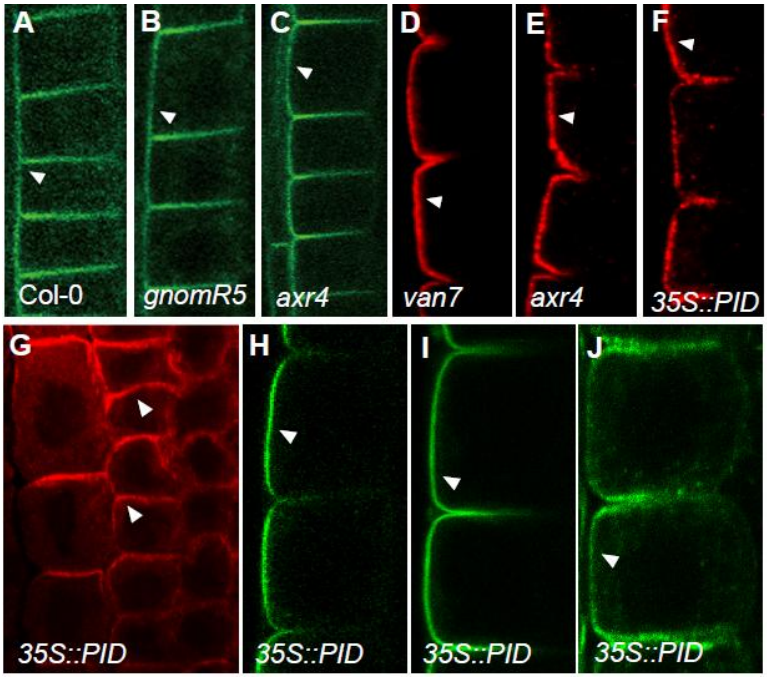

Fig. 4. Distinct molecular mechanism for outer lateral targeting.

Components of the apical or basal targeting are not required for outer polar localization: BOR4-GFP (*A-C*), and ABCG37 (*D-E*) outer localization is not affected in *gnom* (*gnomR5*, *B*; *van7*, *D*), or *axr4* (*C, E*) mutants defective in PIN1 basal localization or AUX1 apical localization, respectively. Outer localization of ABCG37 (*H*), PEN3-GFP (*I*) or BOR4-GFP (*J*) is not affected in *35S::PID* roots in contrast to apicalization of PIN2 in cortex cells (*G*). Immunostainings with anti-ABCG37 (*D-F*), anti-PIN1 and anti-PIN2 (*G*), anti-GFP (*H-J*) antibodies; life cell imaging (*A-C*). Arrowheads indicate polar localization of proteins.

Importantly, also manipulation of the activity of the major apical-basal polarity regulators, protein kinase PINOID [Christensen 2000, Geldner 2003, Friml 2004] and PP2A protein phosphatase [Michniewicz 2007] did not affect the outer localization of BOR4-GFP, ABCG37, and PEN3-GFP (Figure 4 *F-J*; see also Figure S4 *D*). Additionally, other known components of PIN polar targeting does not seem to be involved in outer lateral targeting as demonstrated by unchanged localization of ABCG37 in corresponding mutants (Figure S4). In summary, these data reveal that, outer polar targeting requires distinct molecular components than known apical and basal targeting pathways.

In plants, the necessity of functional interface between plant and environment is obvious but virtually nothing is known about how it is defined at the subcellular level. In this work we examine the localization and the mechanism of polar delivery of few known proteins localizing to the lateral sides of epidermis cells [Ito 2006, Miwa 2007, Strader 2009]. The concomitant localization of these proteins and apical and basal cargos in the same cells, demonstrates that the root epidermal cells possess an additional polar domain, specifically facing the environment We designated this domains the outer polar domain. This domain is defined in peripheral root cell layers independently of the position or apical-basal axis of the given cell. The outer polar targeting requires molecular components and involves cellular mechanisms other than those of the known apical and basal targeting pathways. Thus, plant cells can be characterized by more than two polar domains with corresponding distinct targeting pathways. Transport components for nutrients, hormonal signals, as well as mediators of pathogen defence are specifically targeted to this outer polar domain [Kobae 2006, Stein 2006, Ito 2006, Miwa 2007, Strader 2009]. Thus the outer targeting machinery is presumably of crucial importance to deliver transporters and other cargos, specifically to the outermost cell surfaces of the root, and to functionally define the epithelium-like root-soil interface. The refined characterization of the cellular mechanism and molecular components underlying the outer polar targeting is an important topic for investigations in the coming years and will contribute to our understanding on how the root system interacts with its environment.

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Supplemental Figures

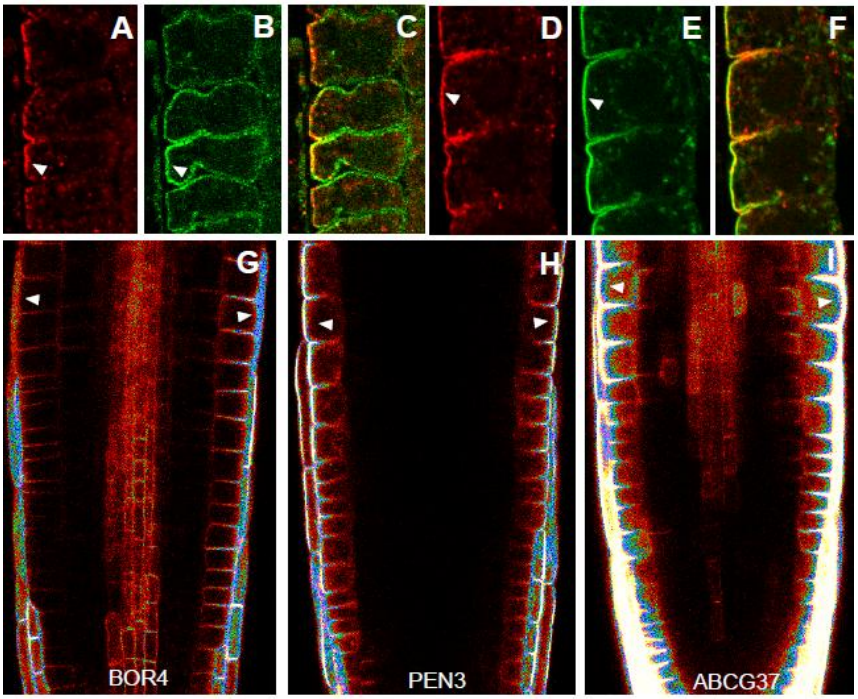

Fig. S1. Colocalizations of outer lateral cargos

(*A-F*) Co-localization of anti-ABCG37 (*A, D*), BOR4-GFP (*B*), and PEN3-GFP (*E*). Merged (*C, F*). (*G-I*) Comparison of signal intensities in *35S::BOR4-GFP* (*G*), *PEN3::PEN3-GFP* (*H*), and *ABCG37::GFP-ABCG37* (*I*) roots reveals weak signal intensity and less pronounced lateral polar localization of BOR4-GFP as compared to GFP-ABCG37 and PEN3-GFP. This suggests that the control of expression under control of strong 35S promoter is not responsible for less pronounced polarity of BOR4-GFP. Immunostainings with anti-ABCG37 antibodies (*A, D*), BOR4-GFP and PEN3-GFP imaging (*B, E*). Life cell imaging with colour-coding of signal intensities (*G-I*). Arrowheads indicate polar localization of proteins.

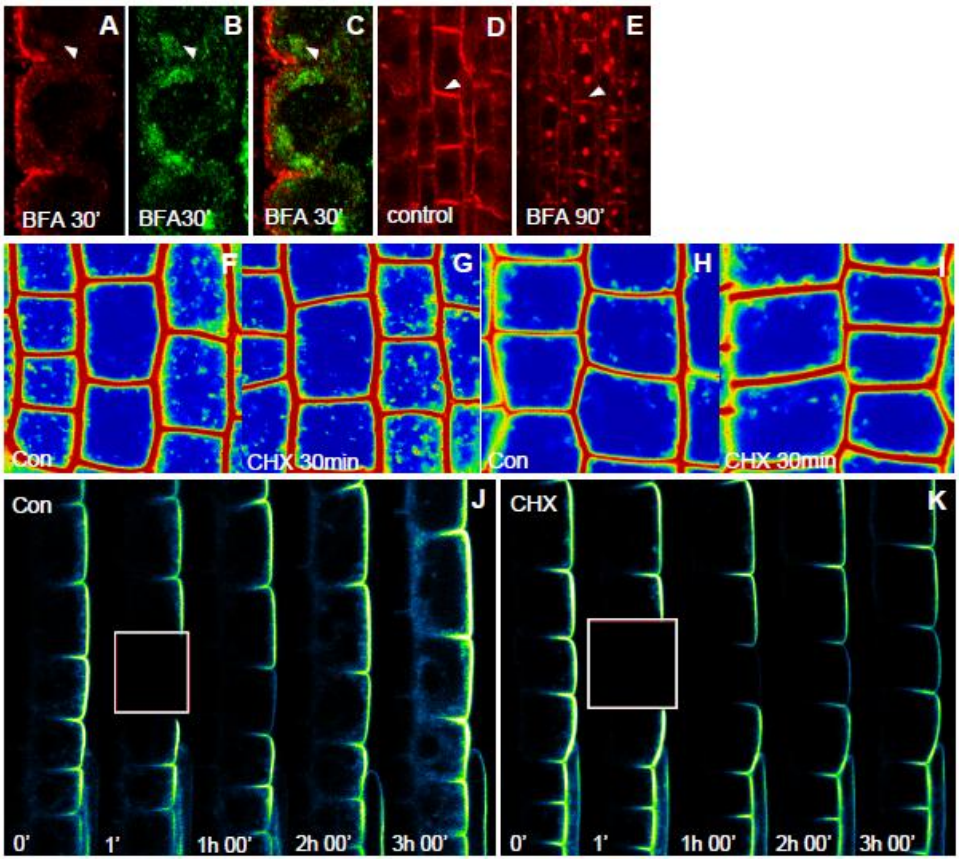

Fig. S2. BFA-sensitive polar targeting and cycloheximide-sensitive secretion

(*A*-*C*) BFA treatment (50 µM, 30 min) leads to strong internalization of the basal PIN1-HA (*B*), but not of laterally localized ABCG37 (*A*); merged (*C*). (*D*, *E*) BFA treatment (50 µM, 30 min) leads to strong internalization of the basal PIN1 in its endogenous expression domain (*E*); untreated control (*D*). Note the decreased plasma membrane PIN1 signal (arrowheads) following BFA treatment. Immunostainings with anti-ABCG37 (*A*, *C*), anti-HA (*B*, *C*), anti-PIN1 (*D*, *E*) antibodies. Arrowheads indicate intracellular aggregations (*A*-*C*) and depletion of PIN1 signal in the plasma membrane (*D*, *E*). (*F*-*I*) Tracking FM4-64 uptake on GFP-ABCG37 (*F*) and PEN3-GFP (*H*) roots without (20 min DMSO / 10 min DMSO + FM4-64) and with cycloheximide (20 min CHX / 10 min CHX + FM4-64) (*G* and *I*). No obvious effects of CHX on endocytosis were observed. (*J*-*K*) FRAP experiments on PEN3-GFP roots (*J*), shows strongly delayed protein recovery and general signal depletion at the plasma membrane in the presence of cyclohexamide. This suggests that constitutive endocytosis still occurs in presence of CHX and that *de novo* synthesised proteins are necessary for signal recovery (*K*). Numbers indicate time points from the bleaching. 1' is a pre-bleach with indicated bleaching area.

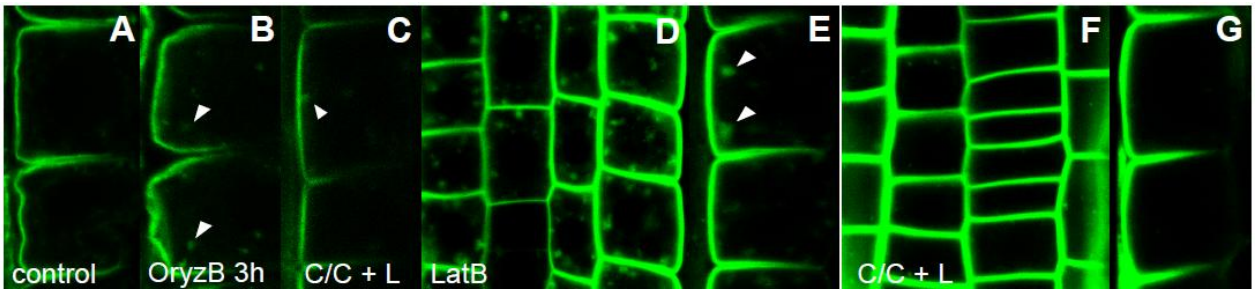

Fig. S3. Involvement of actin and microtubules in secretion to outer lateral domain.

(*A*-*C*) Depolymerization of microtubules by oryzalin B (50 µM, 3 h) treatment leads to very limited intracellular agglomerations of GFP-ABCG37 (*B*), but without effect to its outer polar localization. (*D*-*G*) Depolymerization of actin filaments by latrunculin B (20 µM, 3 h) treatment leads to limited intracellular agglomerations of GFP-ABCG37 (*D* and *E*) and PEN3-GFP (see also Figure 3 *J*. The intracellular signals largely diminish when roots are pre-treated with the protein synthesis inhibitor cycloheximide (50 µM, 30 min) (PEN3-GFP, Figure S3 *C*; GFP-ABCG37, Figure S3 *F* and *G*). Immunostainings with anti-GFP antibodies (*A* and *B*). Life cell imaging (*C*-*G*). Arrowheads indicate agglomeration of proteins.

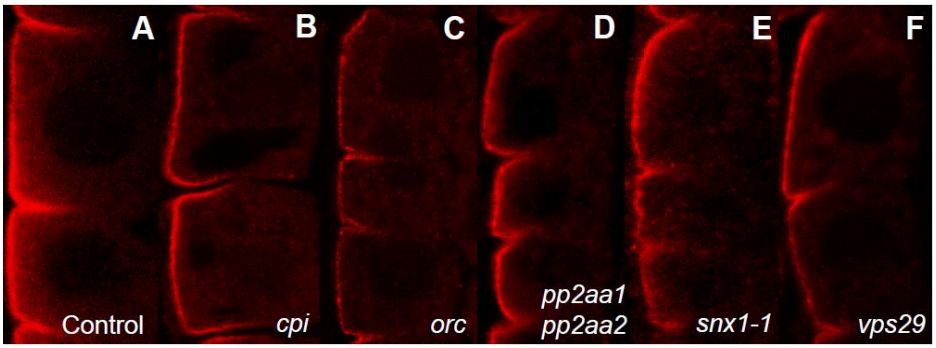

Fig. S4. ABCG37 outer lateral localization in mutants defective in polar PIN localization.

(*A*-*F*) Outer lateral localization of ABCG37 is not affected in established polarity mutants such as *cpi* (*B*) and *orc* (*C*) mutants defective in the membrane sterol composition [see SupRef. 5, 6]; *pp2aa1 pp2aa2* (*D*), defective in PP2A phosphatase that regulates PIN polar targeting [see SupRef. 7]; or *snx1-1* (*E*), and *vps29* (*F*) mutants, defective in PIN trafficking [see SupRef. 8, 9]. Immunostainings with anti-ABCG37 (*A*-*F*). Arrowheads indicate intracellular aggregations (*A*-*C*) and depletion of PIN1 signal in the plasma membrane (*D*, *E*).

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Materials and growth conditions.

Arabidopsis thaliana (L.) Heynh. seeds were sterilized with chlorine gas and stratified at 4°C for 2 days in the dark. Five-day-old seedlings were grown on vertically oriented plates containing *Arabidopsis* medium (AM; half-strength Murashige and Skoog medium, agar, 1% sucrose, pH 5.8) under a 16-h photoperiod, at 22°/18°C.

The following mutants, transgenic plants and constructs have been described previously: *PEN3::PEN3-GFP* [Boutté 2007], *35S::PID* [Benjamins 2001], *axr4-1* [Hobbie 1995], *gnom*^{R5} [Geldner 2004], *orc* [Willemsen 2004], *cpi* [Men 2008], *pp2aa1 x pp2aa2* [Michniewicz 2007], *snx1-1* [Jaillais 2006], *vps29* [Jaillais 2007], *35S::BOR4-GFP* [Miwa 2007]. For *35S::GFP-ABCG37* the ABCG37 genomic fragment was cloned into an appropriate pEPA vector [Dhonukshe 2007]. The fusion construct was then subcloned into binary vector pML-BART [Gleave 1992] and transformed into *pis1-1* mutants.

Treatments.

The seedlings were treated with 50 µM Brefeldin A (BFA; Invitrogen) for 30 min or 90 min; 50 µM cycloheximide (CHX; Sigma) for 30 min or 3.5 h; 50 µM/50 µM CHX/BFA for 30 min; 20 µM Latrunculin B (LatB; Calbiochem) for 3 h; 50 µM CHX for

30 min followed by 50 µM CHX plus 20 µM LatB for 3 h; 50 µM Oryzalin B (OryzB; ChemService) for 3 h; 50 µM CHX for 30 min followed by 50 µM CHX plus 50 µM OryzB for 3 h; 50 µM CHX for 20 min followed by 50 µM CHX plus 8 µM FM4-64 (Invitrogen) dye for 10 min; DMSO for 20 min plus 8 µM FM4-64 for 10 min. All treatments were carried out in AM liquid medium at room temperature in the light and at least in triplicate, with a minimum of 20 roots for each treatment. Control treatments contained an equal amount of solvent (dimethylsulfoxide or methanol).

Localization analysis.

Immunolocalizations in *Arabidopsis* were carried out as described [Sauer 2006]. The following antibodies and dilutions were used: anti-PDR9 [14] rabbit (1:600), anti-PIN1 [Kleine-Vehn 2006] rabbit (1:1000), anti-PIN2 [Benjamins 2001] rabbit (1:800), anti-GFP mice (1:600; Molecular Probes), anti-HA (1:600; SantaCruz), primary antibodies; and ALEXA Fluor 488 anti-rabbit (1:600; Invitrogen), CY3-conjugated anti-rabbit (1:600), and FITC or CY-5-conjugated anti-mouse (1:600) secondary antibodies (Dianova). For triple labelling, the *PIN2::PIN1-GFP2* (in Col-0) transgenic line [Wiśniewska 2006] was used.

Microscopy.

For confocal laser scanning microscopy, a Zeiss LSM5 Exiter and Zeiss 710 where used. Live-cell imaging and FRAP analysis were done with a confocal microscopes (models TCS SP2; Leica and Zeiss 710) equipped with an argon laser (which provides excitation at 488 nm for GFP). For the photobleaching experiment, a region of interest was selected for scans using the Leica (Figure 2) and Zeiss (Figure S2 *J* and *K*), FRAP procedures. GFP images before and after scans were collected. All FRAP analyses were done with the Leica and Zeiss Confocal Software respectively. Images were processed in Adobe Photoshop.

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CHAPTER4

Mechanistic framework for apical, basal and lateral polar localization in Arabidopsis

Łukasz Łangowski, Krzysztof Wabnik, Steffen Vanneste, Satoshi Naramoto and Jiří Friml

Author's Contribution: JF and ŁŁ initiated the project; JF and ŁŁ designed the experiments; ŁŁ carried out most of the experiments; KW performed experiments presented in Figure 4A-C, E,F,H; Figure 8F-H; Figure S5A-H; ŁŁ associated the figures, ŁŁ and SV discussed the results and wrote the manuscript.

SUMMARY

Asymmetric distribution of proteins like PINs, PDRs, BORs and others at a cell's plasma membrane are important determinants of directional auxin flow, nutrients through cells and the cellular response to pathogens. Therefore the mechanisms underlying the polar localization of such proteins are central aspects of plant development. Different polarly localized proteins display specific secretion, trafficking and diffusion dynamics. Here we found that diverse polarities can be achieved by modulation of the same basic mechanisms of polarity generation and maintenance. By FRAP and computer simulations, we found evidence that a preferentially polar secretion is necessary to mimic and maintain real polarity patterns. Besides secretion we also estimated rates of lateral diffusion and super polar exocytosis and implemented them into a computer model that very closely resembles the real signal distribution. Our studies suggest that the polarization and polarity maintaining processes are very dynamic and are fluently altering not only between apical, basal, outer- and inner-lateral domains but also between protein homolog's.

INTRODUCTION

Asymmetric distribution of the proteins is a prerequisite of many cellular processes like cell division, intracellular communication, morphogenesis and differentiation [Grebe 2001]. In animals, cell polarization is achieved by the coordinated activity of remarkably conserved polarity regulators. The first proteins (PAR- partition defective) where identified in a screen for maternal-effect genes that are embryonic lethal in *Caenorhabditis elegans* [Kemphues 1988]. Later, all polarity proteins identified in *Drosophila melanogaster* turned out to have at least one homologue in mammals [Assemat 2008]. In recent years the apical-basal formation of polarity in animals is being intensively studied in *D. melanogaster* and mammalian epithelial cells, where many contributing structural and signaling proteins have been identified. They include mainly apical junctional complexes CRB3/PALS1/PATJ and the Par3/Par6/aPKC and basolateral Scribble/DLG1/LGL1/2 complex [Wodarz 1995; Iden 2008]. Phosphorylation-based mutual exclusion of the Scribble complex and the apical CRB and PAR complexes controls apical–basal asymmetry [Shin 2006]. These proteins not only play an important role in the establishment and maintenance of apical-basal polarity but, also initiate and guide formation of tight junctions. Especially well studied in epithelial cells tight junctions are physical obstacles, which constitute a diffusion barrier for proteins and lipids in the membrane, maintaining distinct compositions of apical and basolateral plasma membrane domains [Iden 2008].

In plants the situation with the polar domains is largely distinct, there are so far four described polar domains [Grebe 2010] and no obvious homologues of the molecular components of animal polarity protein complexes could be found in plants [Geldner 2009; Dettmer 2011]. Furthermore, no structures similar to tight junctions, that delineate polar domains in the plasma membrane of epithelial cells in animals, have been identified in most plant cell types. However plants developed a cell wall, which seems to be crucial for

polarity maintenance in plants, [Feraru 2011]. To control exchange of water and nutrients with the environment and interior, plants have Casparian Strips, ligno-suberic bands deposited on the radial and transverse walls of endodermal cells [Roppolo 2011]. The Casparian Strip represents a physical barrier alike tight junction, however the limited occurrence of this structure to endodermal cells does not favor a major role in a general mechanism of cell polarity maintenance in plants.

In plants there are apical, basal, outer-lateral and inner-lateral polar domains reported. Apical and basal polar domains are already well described, mainly because of intensive research on is the subcellular polar localisation of auxin transporting PIN proteins [Vanneste and Friml 2009; Grunewald and Friml 2010]. PIN's polar localization at the plasma membrane depends on cell type- and PIN sequence-specific factors [Wisniewska 2006]. The subcellular polar localisation of PINs depends to a big extent on the PIN phosphorylation status which is controlled by the serine/threonine protein kinase PINOID (PID) and its counteracting phosphatase PP2A [Friml 2004; Michniewicz 2007; Huang 2010; Zhang 2010]. Phosphorylation of PIN proteins promotes their trafficking to the apical/upper/shootward cell side in the root and shoot apical meristems, whereas their dephosphorylation results in recruitment to the ARF GEF (guanine-nucleotide exchange factors for ADP-ribosylation factor GTPases) GNOM dependent basal/lower/rootward targeting pathway [Geldner 2003; Kleine-Vehn 2009]. Delivered PIN proteins undergo constitutive clathrin-mediated endocytosis from the plasma membrane and recycling [Geldner 2001; Dhonukshe 2007; Kitakura 2011]. The role of these constitutive subcellular dynamics is unclear but it allows to rapidly change PIN localization, to redirect auxin flow in response to internal [Paciorek 2005; Sauer 2006; Scarpella 2006; Wabnik 2010; Friml 2003] and external cues like gravity or light for tropism responses [Kleine-Vehn 2008a; Kleine-Vehn 2008b; Ding 2011; Rakusova 2011].

Besides apical-basal polarity corresponding rather to animal planar polarity [Grebe 2010], also outer and inner lateral polar domains where described in roots with identification of transporters for nutrients such as boron or silicon [Takano 2002; Takano 2005; Miwa 2007].

Comparing to apical and basal domains, the mechanisms underlying outer and inner lateral polar proteins deposition is even less understood. So far, there are no described molecular or pharmacological means that could specifically disrupt this lateral polarity pathways. First glimpses into the cellular mechanism include: actin- and guanine-nucleotide exchange factors for ADP-ribosylation factor GTPases (ARF GEF) dependent secretion of different cargos to the outer polar domain [Łangowski 2010], identification of tyrosine-based sorting signals ensuring inner lateral localization of boron transporter BOR1 [Takano 2010; Grebe 2010] and ARF GEF-mediated endocytic trafficking of BOR1 to the vacuole [Takano 2005].

Despite that our knowledge is progressively increasing, the components and mechanisms underlying polar delivery and molecular regulators of dynamically changing polarity in plants remain poorly characterized. To gain insights into the mechanism of apical, basal and lateral polarity, we performed a systematic comparative analysis of polar delivery in root cells of markers targeted to apical, basal, outer lateral and inner lateral cargos including transporters of hormones (PIN1, PIN2, ABCG37/PIS1; ABCG36/PEN3) and nutrients (BOR1, PIP2). By combining cell biology, genetic approaches and modeling we gained novel insights into secretion, lateral diffusion, endocytic recycling and polarity transition between polar domains.

RESULTS

Evaluation of polarity establishment at apical, basal, outer- and inner-lateral polar domains (Delivery of cargos to polar domains).

To dissect possible similarities and differences between all reported polar domains in respect to secretion, lateral diffusion and super polar recycling, we investigated the subcellular distribution of the polarly localised PIN1, PIN2, PIS1/PDR9/ABCG37, PEN3/PDR8/ABCG36, BOR1 and apolarly localised PIP2 (Plasma Membrane Inrinsic Protein 2) in the *Arabidopsis thaliana* roots via the previously reported transgenic lines *PIN1::PIN1-GFP* [Benkova 2003], *PIN2::PIN-GFP* [Xu and Scheres 2005], *35S::GFP-PIS1* [Růžička 2010], *PEN3::PEN3-GFP* [Boutté 2007], *BOR1::BOR1-GFP* [Takano 2010] and *35S::PIP2-GFP* [Cutler 2000], (Figure 1A-L). The stele-expressed PIN1-GFP showed clear a basal localization within each cell and also to lesser extent lateral signal (Figure 1A). In the epidermis, PIN2-GFP showed intense apical signal and a minor lateral signal that gradually decreased towards the lower cell side (Figure 1C). While, PIN1- GFP and PIN2-GFP delineate apical and basal polarities, ABCG37-GFP and ABCG36-GFP localized to the outer-lateral domain of epidermal cells (Figure 1E and G) and BOR1-GFP clearly localized to the inner-lateral domain (Figure 1I), but also showed a prominent signal maximum at the apical and basal cell sides. As a reference to the polarly localized proteins we have chosen apolar PIP2-GFP (Figure 1K). However, closer investigation revealed some signal asymmetry between polar domains. The strongest signal intensity was measured at the apical/basal domain with the signal gradient increasing towards the outer-lateral domain, the weakest was reported at the inner-lateral side. Interestingly, among all the polarly localized proteins the polarity index was the lowest for PIN1-GFP, which was 10-fold lower that of ABCG37-GFP (which shows the highest polarity index),

(Figure 1M). To evaluate how the polar signal is distributed within its polar domain, we used 3D-reconstructions (x,y,z) of the individual GFP signals and used color-coded fluorescence intensity profiles to evaluate relative protein levels. PIN1-GFP signal showed significant signal enrichment in the inner core of basal cell surface compared to its edges and lateral domains (Figure 1B). Similarly to PIN1-GFP, PIN2-GFP (Figure 1D), ABCG37-GFP and ABCG36-GFP showed a strong enrichment GFP signal at the central zone of their respective polar domains that they decorate (Figure 1D, 1F, 1H), suggesting that polarity establishment of various cargoes at different polar domains shows similar features. Unfortunately, it was difficult to properly evaluate the inner-lateral polarity of BOR1-GFP due to its more transversal localization (Figure 1I and J).

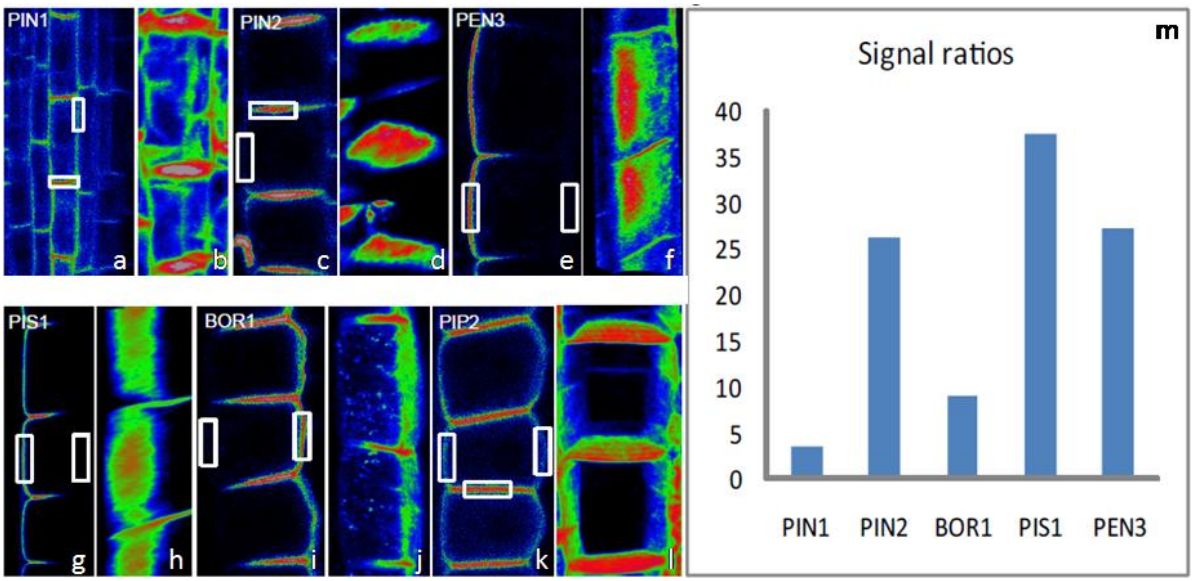


Figure 1. Steady-state localization of various plasma membrane proteins combined with 3D reconstructions.

(*A-B*) Basal localization of PIN1-GFP, (*C-D*) apical localisation of PIN2-GFP, (*E-F*) outer-lateral localisation of PEN3-GFP, (*G-H*) outer-lateral localisation of PIS1-GFP, (*I-J*) inner-lateral localisation of BOR1-GFP, (*K-L*) least polar localisation of PIP2, (*M*) Quantitative polarity index (ratio of polar to lateral or opposite domain according to the white boxes depicted at single scan section of each marker) for steady-state signal intensity. Data are mean and s.e.; n=20-25 roots. All the markers are presented in 2- and 3 dimensional plain (left picture and right picture respectively). 3D pictures illustrate the signal enrichment at different polar domains within single cell. Except PIN1 which is expressed in stele all the markers where analyzed in epidermal cells.

The identification of a central signal maximum in at least 3 different polar domains suggest that common principles underlay polarity in these polar domains. Therefore it is important to measure and understand the differences and overlaps in secretion, lateral diffusion, recycling and degradation between various polarly localized proteins to better polarity generation and maintenance in various polar domains.

Lateral diffusion as a significant factor disrupting polarity maintenance

The plasma membrane is a liquid mosaic in which protein can diffuse. The velocity of this process is determined by membrane fluidity and binding kinetics of the molecules to anchored or slowly moving structures [Chen 2006]. To obtain a better insight into protein diffusion dynamics in apical, basal and outer- and inner-lateral domains we performed fluorescence recovery after photobleaching within a 2 µm membrane region and confocal-based semi-quantitative imaging (Figure 2, Figure S1 and S2). To assess the contribution of lateral mobility and secretion of *de novo* synthesized proteins and recycling in recovery process we used energy and biosynthesis inhibitors (sodium azide, 2-deoxy-D-glucose and cycloheximide) [FRAP, Chen 2006; Men 2008; Boutté 2010], (Figure 2). Signal recovery in treated and nontreated plants wasn't significantly different, suggesting a limited role for secretion and recycling in signal recovery during the time of 5min and 10min after bleaching. Among PIN1-GFP, PIN2-GFP, 35S::GFP:PIS1, PEN3-GFP, BOR1 and PIP2-GFP, PIN2 was the least mobile protein. Comparing to more or less apolarly localized PIP2, PIN2 was diffusing approximately 2x slower (Figure 2B and F). Interestingly, PIN1 and other polarly localized proteins showed diffusion rate close to PIP2 (Figure 2A). This finding reveals that polar localization of the proteins doesn't necessarily correlate with diffusion rate. Therefore, to deal polarity-disrupting dynamic diffusion and maintain

asymmetric distribution some other mechanisms like preferentially polar secretion or immediate recycling seem necessary.

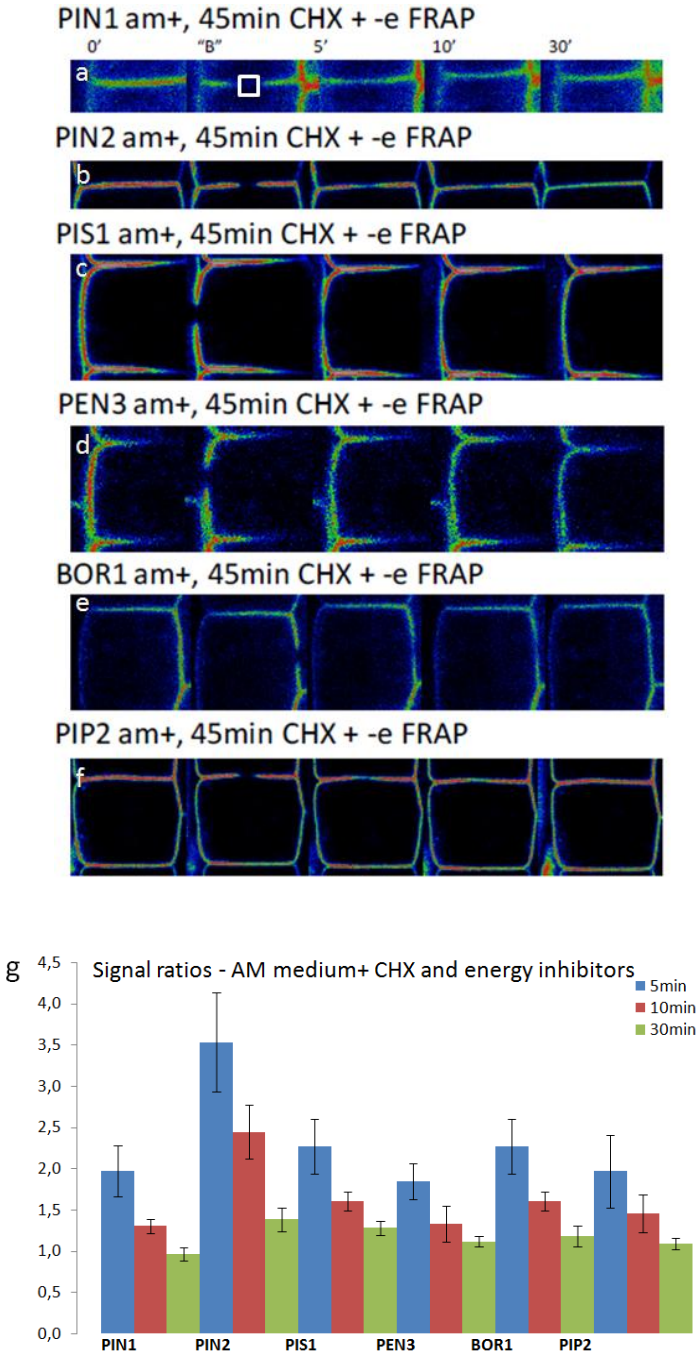


Figure 2. FRAP based lateral diffusion measurements of plasma membrane proteins localizing to apical, basal, outer- and inner-lateral domains within 30min time.

(*A*-*F*) FRAP analyses of (*A*) PIN1-GFP, (*B*) PIN2-GFP, (*C*) PIS1-GFP, (*D*) PEN3-GFP, (*E*) BOR1-GFP, (*F*) PIP2-GFP pre-treated 45min with energy inhibitor, callose inhibitor, biosythesis inhibitor (-e) 0.02% sodium azide, 50mM 2-deoxy-D-glucose and 50µM cycloheximide, respectively. (*G*) Quantitative analyses of experiments (*A*-*G*) showing

signal ratios between mean signal of 2µm bleached and 2µm nonbleached neighbouring region. Signal values of pre- and post-bleach fluorescence intensities data where normalized and mean s.e.; n=3-5 FRAP experiments.

Because apicaly localized PIN2 shows slower diffusion rates than the other tested proteins, we evaluated PIP2 lateral mobility in epidermal cells at transversal and outer-lateral domain. To our surprise PIP2 was diffusing slightly slower at apical in comparison to outer-lateral domains (not shown). Taking into account a neglectible secretion rate in a 10min time window (Figure 2, Figure S1), one can assume that the observed differences may be achieved by differences in plasma membrane composition. Next, in order to evaluate an effect of cell type on lateral diffusion we compared PIN1-GFP mobility in epidermal cells and stele. However, no clear difference in PIN1-GFP lateral diffusion could be observed between the cell types (not shown). Together, these data suggest that properties of the plasma membrane between different polar domains within a single cell may be different and have an influence on polarity maintenance via effects on lateral diffusion.

Secretion is a key player stabilizing asymmetric distribution

After synthesis, proteins get secreted to the plasma membrane. In order to address the contribution initial delivery of polar and non polar cargos to polarity, we photobleached pre-existing green fluorescent protein (GFP)-tagged polar proteins from a group of cells and analyzed its recovery profiles and polarity indexes after 180min (PIN1 200min) (Figure 3, Figure 5, Figure S3). All the proteins showed somewhat different polarity index profiles and recovery rates.

Analysis of the quantitative polarity indexes (generated as presented in Figure 1, basal to lateral PIN1-GFP (Figure 3A, B, E), apical to outer-lateral PIN2 (Figure 3 C, D, F), outer-lateral to inner-lateral PIS1, PEN3 (Figure 4), inner-lateral to outer-lateral BOR1

(Figure S3A and B)) shows that the signal intensity at the polar sides in early stages of recovery is persistently growing revealing strong influence of secretion on polarization. Consistent with its lateral diffusion rate, the polarity index of PIN1-GFP stabilized after about 60min after bleaching indicating that the initially secreted pool of basaly localized protein starts to arrive at the lateral domain via lateral diffusion. Between 60-105min, the polarity index reached its maximum as a consequence of an added effect of recycling which amplifies the signal at the basal domain saturating it.

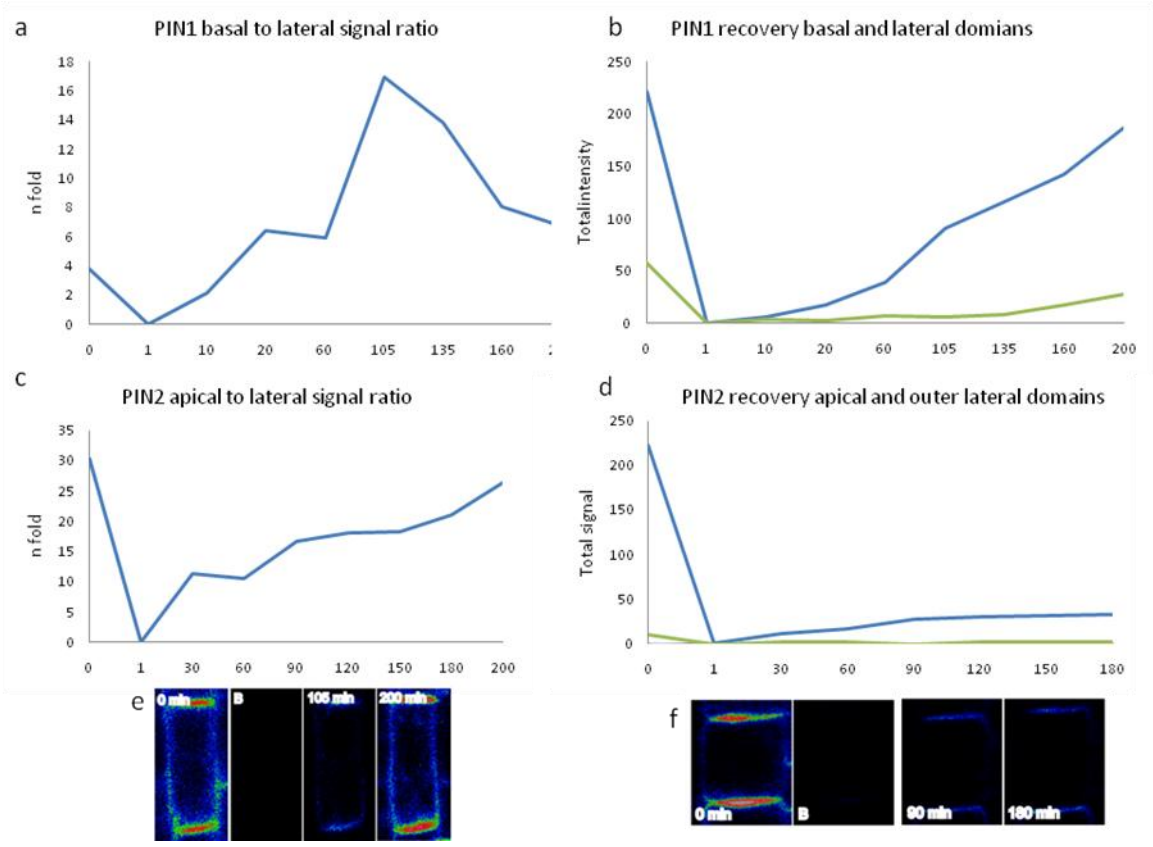


Figure 3. PIN1-GFP and PIN2-GFP targeting to the plasma membrane after complete photobleaching.

(*A*) Quantitative polar index of PIN1 based on (*B*) signal intensity measurements in time at the basal (blue line) and lateral domains (green) after bleaching of the whole cell, shows different progress curve pattern comparing to (*C*) quantitative polar index of PIN2 obtained from (*B*) signal intensity measurements in time at the apical (blue line) and lateral domains (green). One of the reasons is different recovery rate. (*E*) PIN1 shows approximately 80%, (*F*) PIN2 shows approximately 17%. Data are mean, n=4. According to FRAP results showing specific recovery at the polar domains we assume that PIN1 and PIN2 are preferentially polarly secreted. This notion is further supported by the computational

simulation integrating other experimental data like lateral diffusion or super polar exocytosis.

This matches the order of magnitudes that were estimated for recycling of intracellularly accumulated PIN1-GFP after inhibition of exocytosis [Dhonukshe 2007; Kleine-Vehn 2008a]. From 105min after photobleaching onwards, the polarity index didn't increase anymore and started to drop due to two reasons (Figure 3A, B, E). First, increase of the signal at the lateral domain by delayed in time protein lateral diffusion. Second, progressive signal intensity saturation at the polar domain reaching the limits of the dynamic range of the image acquisition settings. From that point, only the signal at the lateral side can increase leading to the saturation of the system and stabilization of the polar index close to the steady-state level.

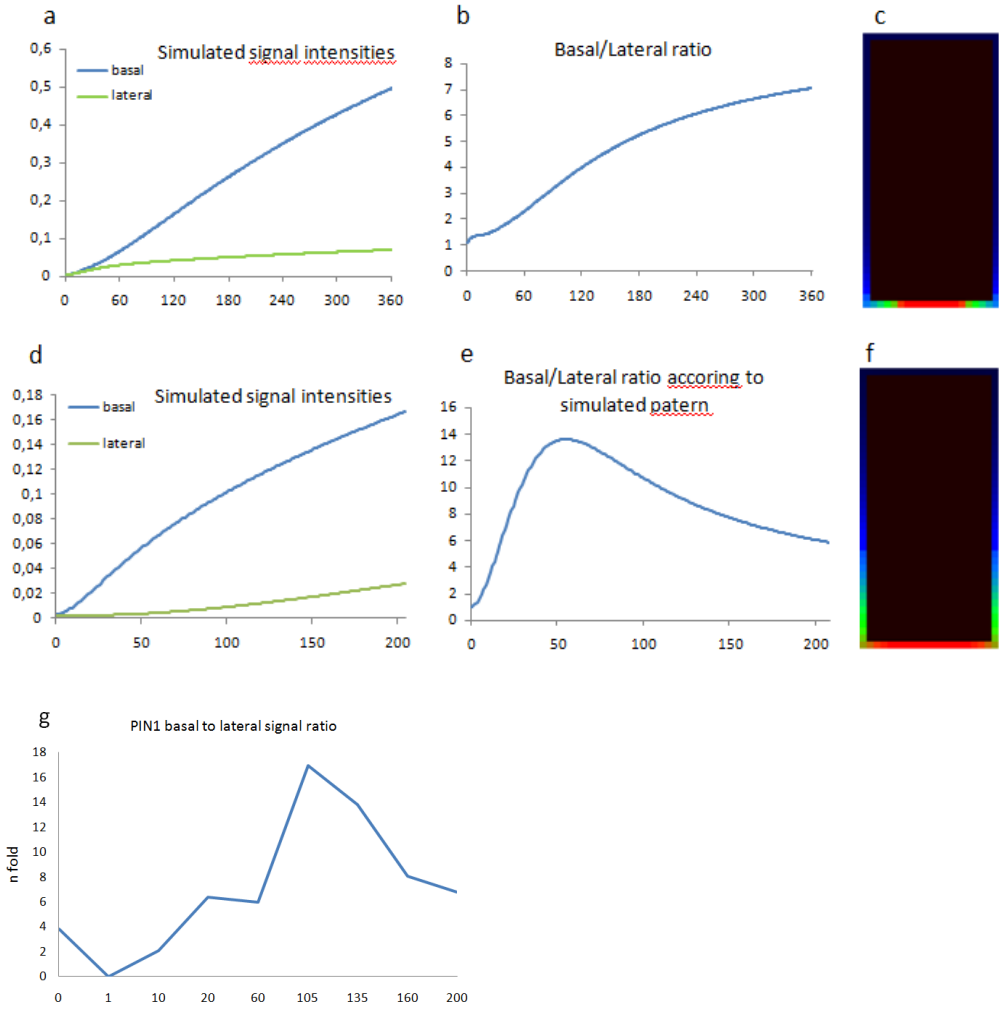


Figure 4. Computational simulation of secretion manner to plasma membrane of PIN1 based on assumptions and experimental data.

In order to test whether the asymmetric distribution of PIN1 at the plasma membrane (PM) occurs in non-polar or preferentially polar fashion we created two models integrating experimental data and hypothetical assumptions. The simulation mimics the FRAP experiment, more specifically befor starting simulation model resembles a bleached cell. Once the it starts all integrated processes (protein synthesis, secretion, lateral diffusion, endocytosis, degradation and recycling) are initiated. In both models, representing (*A, B* and *C*) non-polar secretion and preferentialy polar secretion (*D, E* and *F*) we used the same method as in Figure 3 to generate the polar index. In time we measured the signal intensity at the (*A* and *D*) basal (blue line) and (*A* and *D*) lateral domains (green) and generated (*B* and *E*) quantitive polar index. A model assuming (*D, E* and *F*) preferentially polar secretion shows different progress curve pattern than the model assuming the (*A, B* and *C*) non-polar secretion. Interestingly, the newly proposed model very much resembles (*G*) a PIN1 pattern obtained from FRAP experiments and signal intensity measurements (Figure 3).

In order to further test whether the asymmetric distribution of PIN1 at the plasma membrane (PM) occurs in non-polar or preferentially polar fashion we created two models integrating experimental data and hypothetical assumptions (Figure 4, Figure S4). The model represents a single cell. For computational reasons, we represented the plasma membrane as a sequence of discrete membrane fragments each of 1x1 micron size. The lateral cell sides were considered a 2-fold longer than that of apical or basal cell sides to mimic geometry of root stele cells. The intracellular membranes were approximated by one single endosomal compartment that represented the common intracellular pool of PIN proteins. First model (current) assume that the proteins are randomly secreted to the PM where undergoes modification, what initiates polar sorting of the endocytosed cargo and in consequence polar recycling [Dhonukshe 2010]. Another assumption is that the lateral diffusion of PIN1 is relatively low, the parameter values were estimated from experimental data used in recently published work [Kleine-Vehn and Wabnik 2011]. The second model (newly proposed) assume that the proteins after biosynthesis are modified, sorted at TGN and subsequently preferentially polarly secreted. Second assumption differing the new

model from current is that protein lateral diffusion parameter was proportionally increased according to experimentally obtained data (Figure 2, Figure S1). Besides different assumption about secretion manner and integrated experimental data concerning lateral diffusion there is a number of parameters which are common for both models: secretion rate to the polar domain, polar delivery of the cargo to the central core, endocytosis rate, degradation rate, immediate recycling (faster than secretion rate). All above mentioned parameters where used in recently published model [Kleine-Vehn and Wabnik 2011].

Intriguingly, PIN2-GFP which evidently shows polar localization in steady state, accordingly to computer simulation modeling PIN1 dynamics exhibits a recovery profile reflecting the behavior of nonpolarly secreted protein (Figure 3). One possible explanation is that low synthesis and secretion rate impedes the proper signal ratios measurements (Figure S3D). However, once the signal intensity at lateral domain reaches a measureable level, stays relatively low in comparison to apical domain. Another option is influence of very low protein lateral diffusion of PIN2 on protein dynamics in plasma membrane [Kleine-Vehn 2011; Łangowski Chapter 4]. According to the tests performed with model, decrease of protein lateral diffusion combined with active recycling [Kleine-Vehn 2011; Łangowski Chapter 4] stabilizes polarly secreted proteins at the middle core of polar domain and reduces signal intensity at lateral domain (Figure S4). This observations suggest that in order to estimate the meaner of secretion of different polar markers just one common model is not sufficient. It's necessary to study each protein individually in respect of the recovery rate, lateral diffusion, recycling and secretion contribution.

PIS1-GFP and PEN3-GFP localizing to the outer lateral domain display very sharp polar localization. Their high recovery rate (Figure S3D), relatively high protein lateral diffusion like in case of PIN1 (Figure 2G), intensive secretion and low recycling rate

[Łangowski 2010], suggest important role of trafficking and sorting to the vacuole and intensive polar secretion (Figure 5).

Localized to inner lateral domain BOR1-GFP represents the least polar marker among tested. However according to the obtained results it seems that BOR1 is also polarly secreted to the inner lateral domain (Figure S3A and B). On the other hand one have to take into account strong signal accumulation in the steady state at the apical and basal sides and rapid signal recovery after bleaching within these domains, which may have an effect on plasma membrane BOR1 dynamics (Figure 1I and J, Figure S3A and B).

In summary, our data suggest that polarly localized PIN1, PIN2, PIS1, PEN3 and BOR1 may be preferentially polarly delivered to specific domains. On the other hand, apically localized proteins such as PIN2-GFP, undergoing modifications [Friml 2004; Michniewicz 2007] or polarized to lower extend BOR1, defining inner lateral domain, may use different mechanisms for polarity establishment and maintenance.

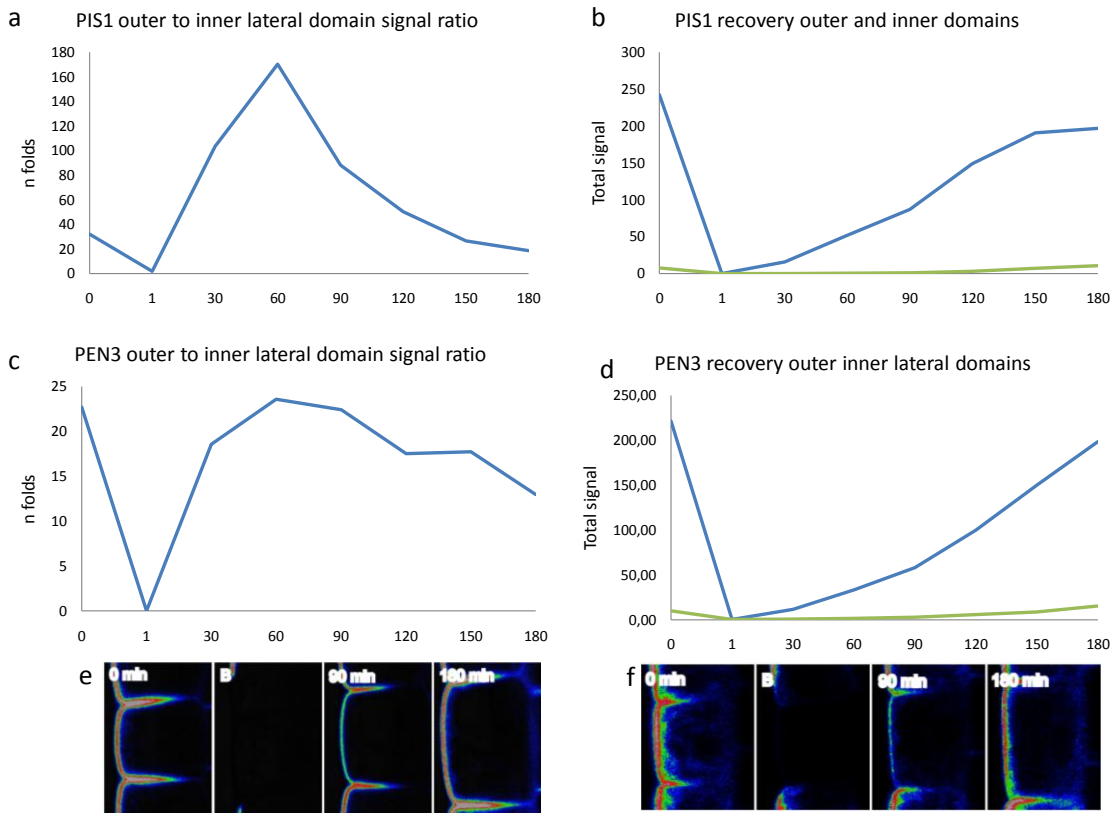


Figure 5. PIS1 and PEN3 secretion patterns suggest polar secretion to the outer-lateral domain.

(*A* and *C*) Quantitative polar index of (*A*) PIS1 (*C*) and PEN3 based on (*B* and *D* respectively) signal intensity measurements in time at the outer lateral (blue line) and inner lateral domains (green) after bleaching of the whole cell, shows similar progress curve pattern comparing to already described PIN1 (Figure 3). Taking into account the computer simulations results for PIN1, it seems that outer lateraly localized PIS1 and PEN3 are also preferentially polarly secreted. (*E and F*) Recovery rates after bleaching of the whole cell of (*E*) PIS1 and (*F*) PEN3 are relatively similar to PIN1, all the proteins show high recovery rate in 180min time which is approximately 80%. Data are mean, n=4-8. The polarity indexes of PIS1 and PEN3 show different values but similar patterns to PIN1 what further supports the assumption, that the preferentially polar secretion is the common mechanism of cargo delivery to the plasma membrane.

Endocytosis-dependent super polar recycling.

Previously it was reported that super-polar PIN2 localization is defined by a polar exocytosis/delivery mechanism [Kleine-Vehn 2011]. Besides the apical domain (PIN2), super-polar localizations could also be detected at other domains. To test whether the same mechanism of super-polar secretion and recycling occurs at lateral polar domains we photobleached the entire outer-lateral (PIS1-GFP) and inner-lateral (BOR1-GFP) cell sides and subsequently followed its recycling and secretion -based recovery within 15–45 min. PIS1 as well as BOR1 in the single image plane didn't show a clear signal enrichment in the center of the domain (Figure 6A and B). However, z-stack imaging (0.5 µm steps) of whole root epidermal cells after 45 min from bleaching revealed that cargo delivery to the inner core of polar domain also occurred for PIS1 and to lower extend for BOR1 (Figure 6A and B). What may suggest that the super-polar cargo delivery to the plasma membrane is a common mechanism maintaining asymmetric protein distribution.

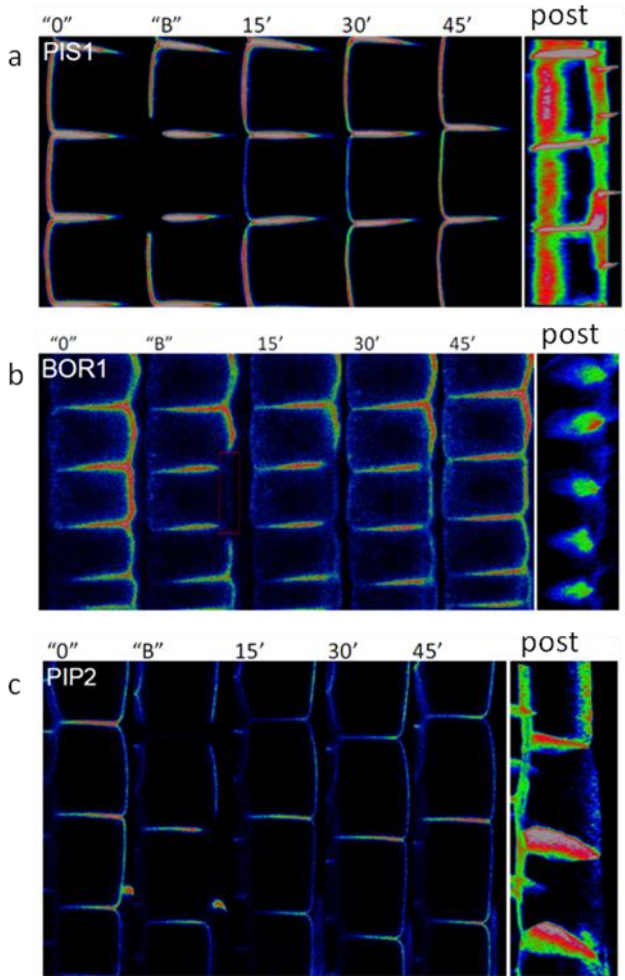


Figure 6. Medial, single scan sections of PIS1-GFP, BOR1-GFP and PIP2.

Pre-bleached cells of PIS1-GFP (outer-lateral domain) and BOR1-GFP (inner-lateral domain) in endodermal cells doesn't show super polar localization of the markers, importantly after photobleaching the signal seems to recover equally at the whole length of the domain (*A and B*). However, 3D reconstructions 45min after bleaching show enrichment of the signal in middle core of the domain for PIS1 and BOR1 (*A post and B post*). PIP2 bleached at the transversal domain shows gradient signal intensity from the surface of the root towards the inner domain suggesting differential egzocytosis within single domain or specific cargo-dependent protein retention (*C post*).

Polarity maintenance.

The importance of the cell wall:

Recently, the cell wall has been implicated as an important factor as a major structure for cell polarity [Feraru 2011].

To address the role of the cell wall as a polarity regulator not only for apical and basal but also inner- and outer lateral domains, protoplasting was performed on GFP-fused markers with different polar localization: outer-lateral PIS1-GFP, PEN3-GFP, inner-lateral BOR1-GFP and both apical PIN2-GFP and PIP2-GFP as a control. Polar localization of all tested proteins was rapidly lost after protoplasting and became uniformely distributed along the plasma membrane of the protoplast (Figure 7F-J). These results demonstrate, that the cell wall is indeed indispensible for polarity maintenance of all polar domains.

To test the hypothesis of plasma membrane vs. cell wall interaction, manintol-induced plasmolysis was used to demonstrate the stability of PINs at the cell wall connection [Feraru 2011]. Therefore, in order test if the same mechanism exists for the lateral domains, we mimicked this experimental set-up using markers localized to outer- (PIS1-GFP, PEN3-GFP) and inner-lateral (BOR1-GFP) domains. The Hechtian strands, indicative of plasmolysis could be observed after 20 min of partial degradation of the cell wall by a protoplasting solution lacking cellulase (Figure 7A-E). The treatment resulted in a good separation of the plasma membrane from the cell wall while preserving of the tissue context. For all analyzed markers the signal could be observed on the Hechtian strands and at the surface of the cell wall where the strands where attached. Interestingly, single plane imaging and 3D reconstructions of all tested lines revealed much stronger protein deposition at the cell wall in case of polar markers in comparison to apolar PIP2 protein (Figure 7A-E and K-N). The highest deposition displayed PIN2-GFP protein which seems to be organized in clusters [Kleine-Vehn 2011; Łangowski Chapter4]. This suggest that the cell wall has a stabilizing effect on the protein localization and mobility what lies in a line with lateral diffusion data.

Overall these data confirmed the association of all the plasma membrane proteins with the cell wall. For some polar markers it may have a great importance, not only for the polarity establishment but also maintenance as a factor that limits lateral diffusion.

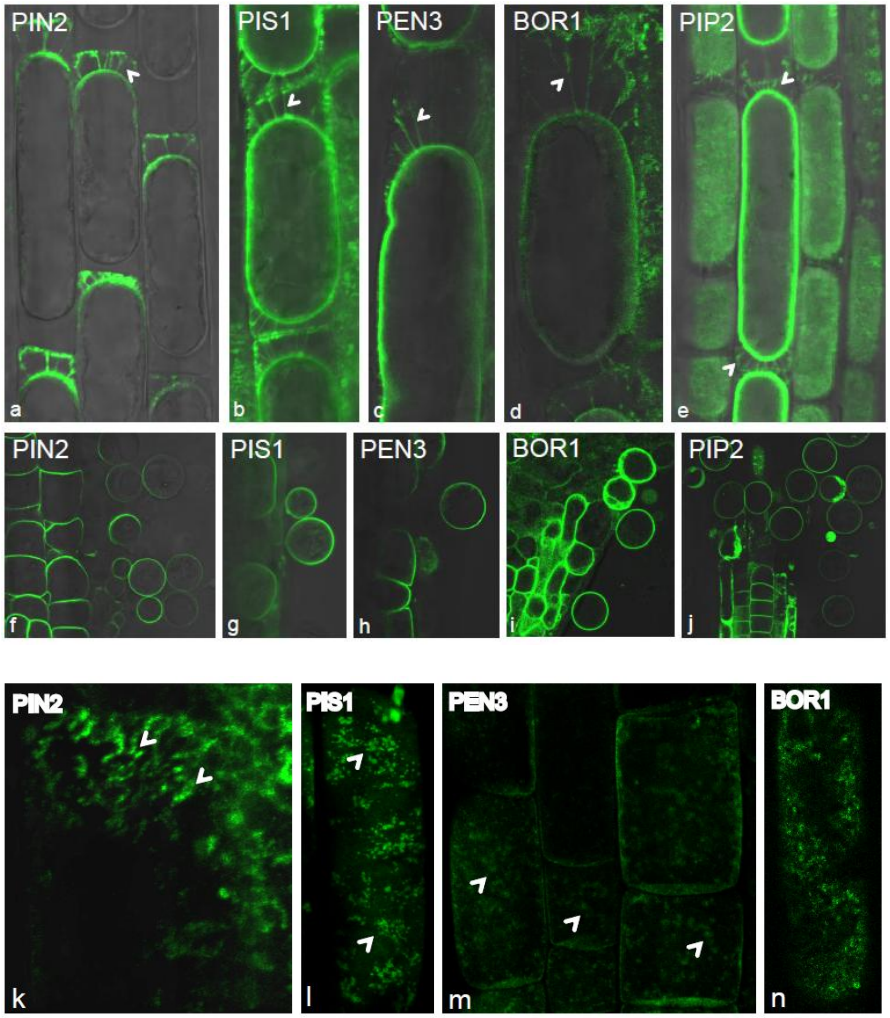


Figure 7. Cellulose-based connections between plasma membrane and cell wall maintain proteins polarity.

(*A*-*E*) Mannitol-induced plasmolysis combined with partial degradation of the cell wall by macerozyme reveals that all tested plasma membrane markers localizing to different polar domains PIN2 (apical), (*A*); PIS1 (outer-lateral), (*B*); PEN3 (outer-lateral), (*C*); BOR1 (inner-lateral), (*D*), PIP2 (apolar), (*E*) are connected by Hectian Strands to the cell wall (*A*-*E*). Entire digestion of the cell wall, protoplasting shows immediate polarity loose of all incubated polar markers (*A*-*E*). 3D reconstructions of PIN2 (*K*), PIS1(*L*), PEN3(*M*) and BOR1 (*N*) markers after plasmolysis shows characteristic clusters at the cell surface, which most likely are the places where the Hectian Strands "touch" the cell wall and proteins are anchored (*K*-*N*). This experiments show that connections between plasma membrane and cell wall are not reserved for polarly localized proteins. However, polar markers reveal higher accumulation of the proteins at the cell wall.

Polarity maintenance: The importance of clusters:

In recent years the discussion about the lipid rafts in animals and microdomains/clusters in plants is very vivid. In fact neither in animals nor plants the nature and importance of the "dense lipid domains" was sufficiently proven

Recently it was shown that PIN1 and PIN2 are not evenly distributed at their polar domains forming so called "clusters" [Kleine-Vehn 2011]. Essentially the data are visualizing the accumulation of the GFP-fused PIN2 and ectopically expressed PIN1 protein over the polar membrane in epidermal cells. We decided to test the "clustering" of other polarly localized proteins in the epidermis like ABCG37, BOR1 and as a reference we used PIN2, PIP2 and PIN1 in stele. PIP2 should not display clustering [Kleine Vehn 2011]. While the clusters were relatively easy to observe for PIN2, we were unable to observe such "clustering" pattern for any of the other lines (Figure 8A-H). That would suggest that, clustering could be specific at the various domains or that this is a protein-specific feature.

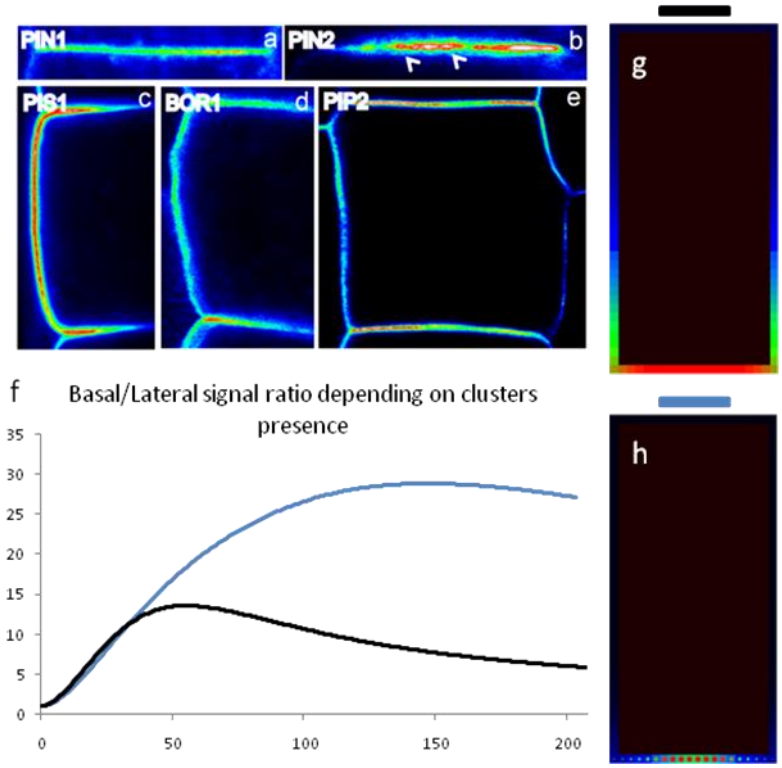


Figure 8. Protein clustering promotes super polar protein localization.

(*A*-*E*) Live imaging on PIN1(*A*), PIN2(*B*), PIS1(*C*), BOR1(*D*) and PIP2 (*E*) revealed that protein "clustering" is easy to observe only in case of PIN2. Interestingly, non of polarly localized proteins including PIN1 did not show obviously heterogeneous plasma membrane signal. This observation corresponds to lateral diffusion rate of PIN2 and other tested proteins, suggesting corelation between low lateral diffusion and protein clustering. Basing on described (in the main text) cell model assuming preferentialy polar secretion we simulated the signal recovery (*G* and *F*) without clusters (black line) and (*H* and *F*) with clusters (blue line). Clustering is defind to immobilize the cargo decreasing an amount of freely diffusing protein. (*F*) Increase of protein retention leads to dramatic change in polar index pattern (from black to blue line) resembling the one experimentaly obtained for PIN2 (Figure 3C). This suggests that PIN2, which displays polar index pattern similar to one obtained from the model assuming non-polar secretion (Figure 4B), seems to be preferentially polarly secreted.

Establishment of polar domains in development

In the last ten years our knowledge about plants polarity determination, establishment and maintenance have significantly increased. From early development onwards, cellular polarities are apparent via the highly organized pattern of cell division. Indeed, polar domains defined by PINs have previously been described in detail for early embryogenesis

[Friml 2003a; Friml 2003b; Blilou 2005]. However, it is not clear when and where the lateral polar domains are established. Therefore, we decided to check when and where lateraly localized markers polarize during embryogenesis and lateral root organogenesis. Up to torpedo stage we could not observe any expression of ABCG37, suggesting very low expression level (Figure 9A). At the torpedo stage we could observe ABCG37 localization in cotyledons, but not in the primary root (Figure 9C). In contrast, a close homolog of ABCG37, a PEN3::PEN3-GFP (ABCG36-GFP) showed a basal localization in root cells in the early torpedo stage followed by a transition to the outer-lateral domain in later stages (Figure 9G, I, K). Originally localized at the inner-lateral domain BOR1::BOR1:GFP didn't show any unexpected signal transitions. The first detectable signal of BOR1 was reported in heart stage in quiescent center (QC), later on in torpedo stage signal was beyond detection (Figure 9M and G).

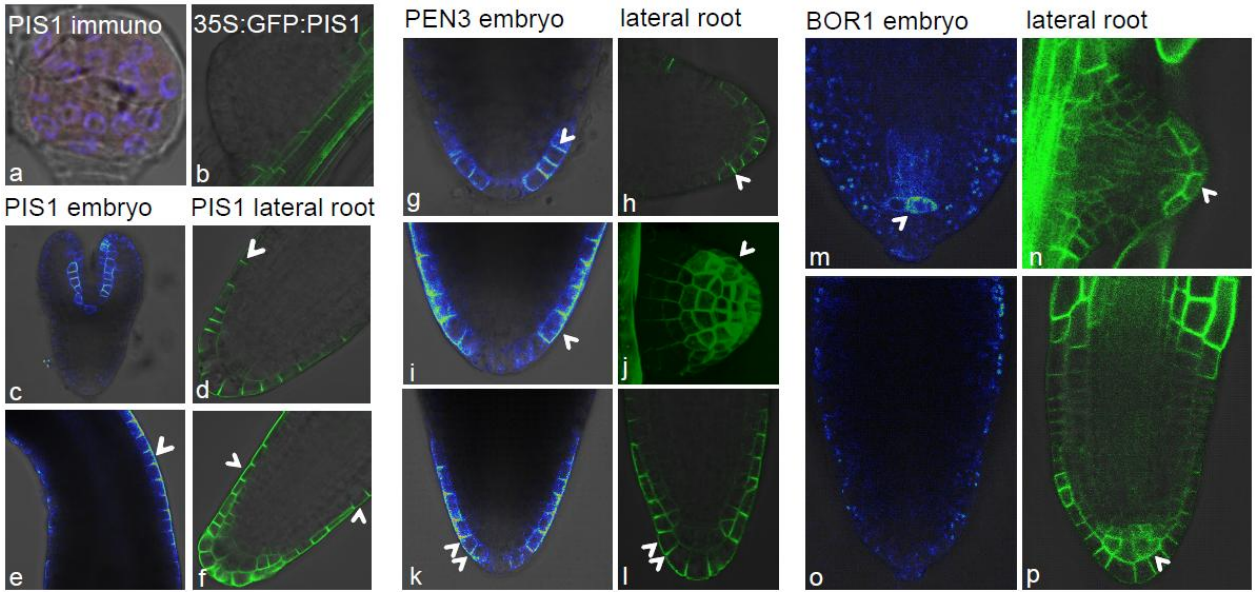


Figure 9. PIS1-GFP and PEN3-GFP show polarity transition during embryogenesis and lateral roots organogenesis.

In parallel we tested the polar localization of PIS1, PEN3 and BOR1 in embrygenesis and emerging lateral roots organogenesis. PIS1 showed very low expression level in emerging lateral roots (*B*) and was beyond detection range in embryo globular stage (tested by immunostaining and live imaging of 35S::GFP:PIS1), (*A*). In torpedo stage PIS1-GFP showed limited expression in cotyledons (*C*). However, in late torpedo stage the signal was

well visible at the outer-lateral domain of epidermis (*E*). Interestingly, PIS1-GFP localization in young lateral changed from clearly basal to basal and lateral in older lateral roots (*D and F*). Similar situation was observed in case of PEN3-GFP which was initially expressed at the basal domain of the cells in early torpedo stage and emerging lateral roots, while in the later stages the signal was strongly visible at the outer-lateral and basal side in both embryo and lateral roots cells (*G-L*). Expression and localization studies of BOR1-GFP didn't reveal any alterations in protein polar localization. BOR1 showed very low expression level in torpedo stage, specifically at QC (*M and O*) in comparison to emerging and young lateral roots (*N and P*). Arrowheads indicate the polar localization of the proteins.

Also during post-embryonic organogenesis, polarities need to be (re)-established. ABCG37 expression in emerging lateral roots (LR) was very low, but later on in development has been continuously increasing (Figure 9B, D, F). In the older LR ABCG37 predominantly distributed to the basal domain polarizes at outer lateral domain (Figure 9D and F). Similar situation was observed in case of ABCG36 which initially targeted to the basal cell side, while in the older LR displayed outer lateral localization (Figure 9H, J, L). Overall these data suggest, that protein polarity may dramatically change within a short developmental time-frame as observed for ABCG36/PEN3 and ABCG37/PIS1. This suggests that plant cells have an number of molecular polarity regulators which are developmentally controlled.

Discussion:

Asymmetric protein distribution is a fundamental aspect of many developmental processes [Grebe 2001]. At the single cell level either it can be defined as a structural asymmetry or asymmetry in localization of intracellular molecules [Dhonukshe 2005]. Both asymmetries interrelate with each other allowing adequate organization of the molecules and prompt response to internal and external cues. In animals the cell polarization is achieved by coordinated activity of molecular determinants including apical junctional complexes CRB3/PALS1/PATJ, Par3/Par6/aPKC and basolateral

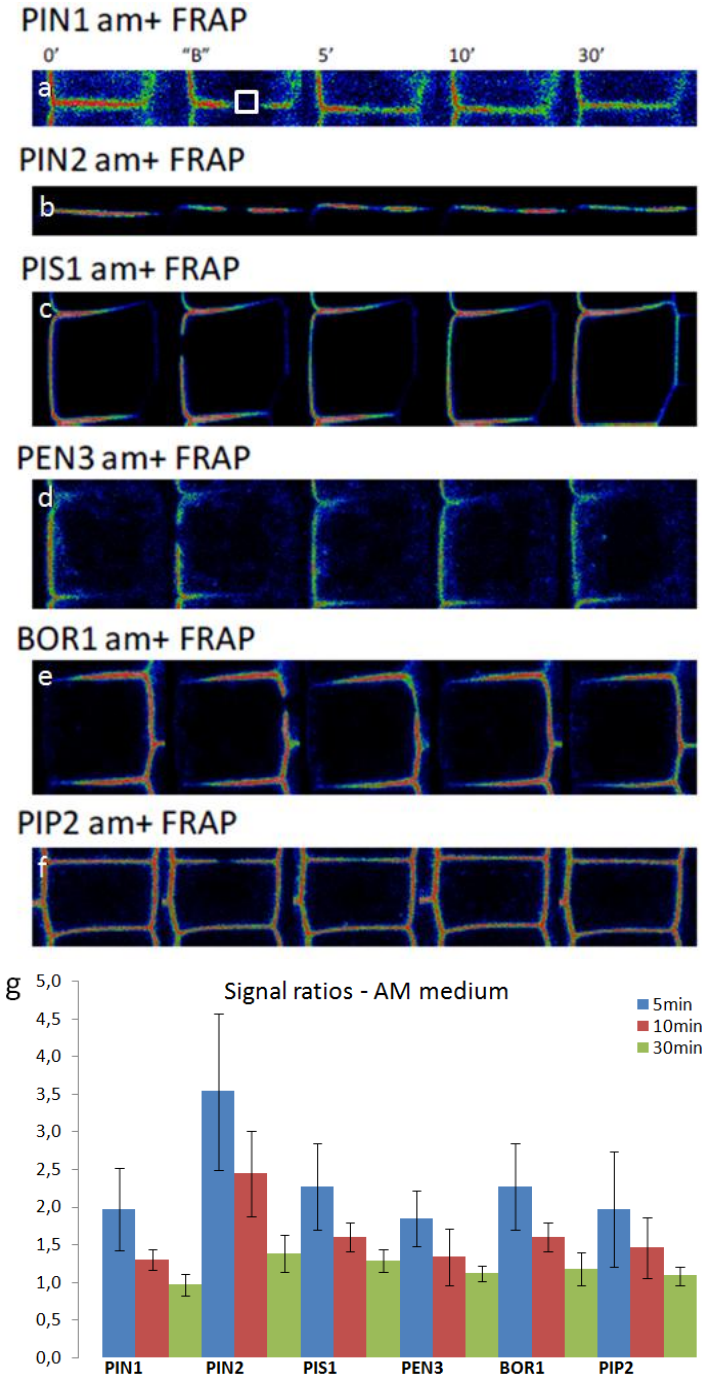
Scribble/DLG1/LGL1/2 complex [Wodarz 1995; Iden 2008], combined with polarity maintening role of physical barrier, tight junction separating apical and basaolateral domains. In plants the molecular components determining asymmetric distribution of the proteins to four described polar domains remain largely unknown. However, the mechanisms underlying the cell polarity are progressively increasing. It has been reported that initial secretion of newly synthesized PIN proteins is non-polar, highlighting the key role of endocytic recycling in establishment of polar protein deposition at the specific cell side [Dhonukshe 2008, Dhonukshe 2010]. It has been also showed, that dynamic PIN polarity maintenance in plants depends on an interweaving mechanism including super-polar delivery to the center of the polar plasma membrane domain, protein recruitment to clusters in the plasma membrane that limits lateral cargo diffusion and a spatially restricted polar endocytosis [Kleine-Vehn 2011]. Here, we present a comparative analysis of different polar markers localizing to apical, basal, outer lateral and inner lateral domains in respect of secretion, lateral diffusion, super-polar exocytosis and polarity maintenance role of cell wall. Our results based on FRAP analysis and computer model simulating protein dynamics within the cell revealed that (i) initially secreted protein reach the plasma membrane in preferentially polar way, (ii) protein lateral diffusion alters from protein to protein and has a prominent influence on polar protein localization, (iii) super-polar cargo delivery seems to be a common mechanism establishing asymmetric protein distribution. Besides that our studies indicate that most likely all plasma membrane localizing proteins are connected with the cell wall in different extend. Furthermore, we demonstrate, that protein polarity may dramatically change within a short developmental time-frame suggesting, that plant cells have an number of molecular polarity regulators which are developmentally controlled.

Various polar markers shows different secretion, lateral diffusion, recycling and degradation rates, computer simulations confirm that the combination of these processes provides a robust mechanism for polarity maintenance in plant cells revealing that preferentially polar secretion is a key regulator establishing and maintaining polarity.

According to FRAP results addressing the issue of protein mobility within the PM we have observed that PIN1 and other polarly localized proteins, (except PIN2), showed diffusion rate close to PIP2, revealing that polar localization of the proteins doesn't necessarily correlate with low diffusion rate. Therefore, to deal with polarity-disrupting dynamic diffusion and maintain asymmetric distribution some other mechanisms like preferentially polar secretion or immediate recycling seem necessary. This observations correspond to protein organization in so-called "clusters" most likely limiting protein mobility. While the clusters are easy to observe for PIN2, we were unable to observe such "clustering" pattern for any of the other lines suggesting that, clustering could be specific at the various domains or that this is a protein-specific feature.

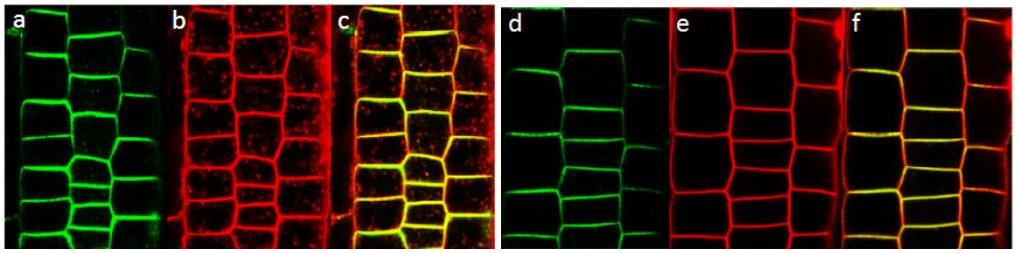
Next to molecular mechanisms and plasma membrane heterogeneity it has been shown that cell wall is a prominent structure ensuring cell polarity [Feraru 2011]. Obviously one could discuss, whether the digestion of the entire cell wall and loss of the tissue context by protoplasting result in a pleiotropic effect, and in concequence loss of polarity. However, *repp3* a cell wall synthesis mutant, defective in proper PIN1-HA localization gives a strong credit to the extracellular matrix as a polarity keeper.

Supplementary information:



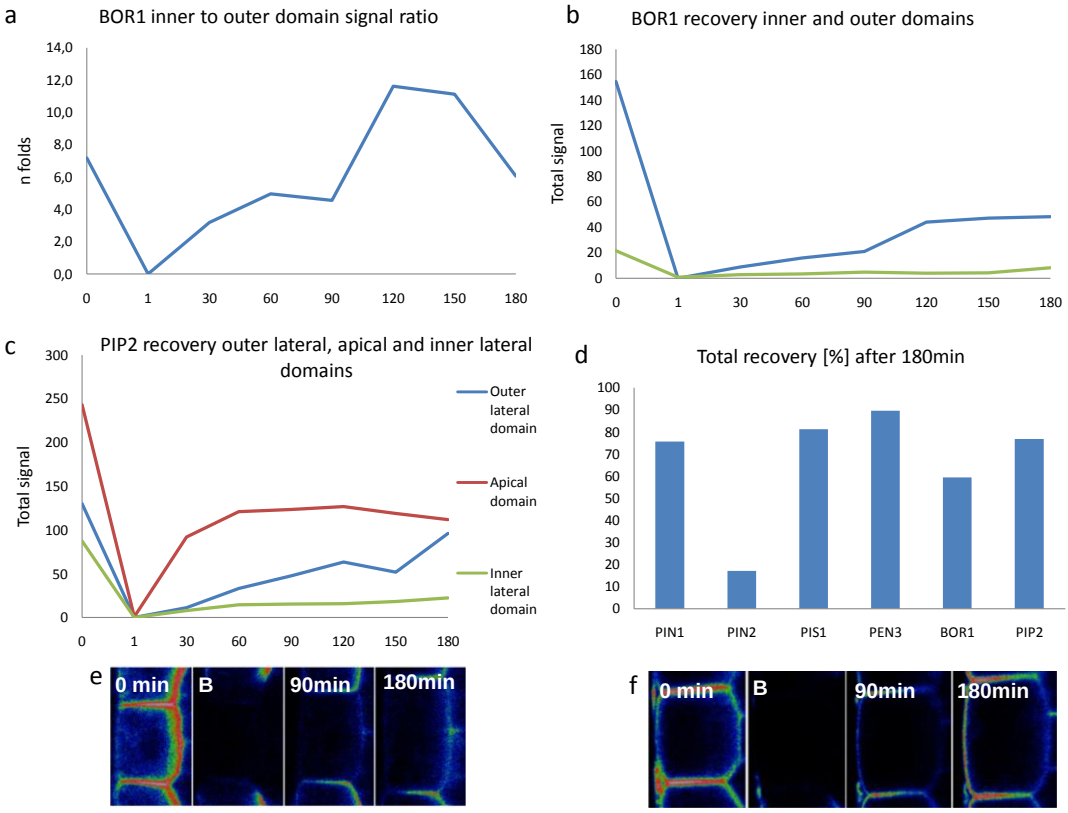
SFigure 1. FRAP based lateral diffusion measurements of plasma membrane proteins localizing to apical, basal, outer- and inner-lateral domains within 30min time.

FRAP analyses of (A) PIN1-GFP, (B) PIN2-GFP, (C) PIS1-GFP, (D) PEN3-GFP, (E) BOR1-GFP, (F) PIP2-GFP (G). Quantitative analyses of experiments (A-G) showing signal ratios between mean signal of 2µm ROI and 2µm nonbleached neighbouring region. Signal values of pre- and post-bleach fluorescence intensities data where normalized and mean s.e.; n=3-5 FRAP experiments.



SFigure 2. Control treatment with energy inhibitors in order to test the physiological processes occurrence.

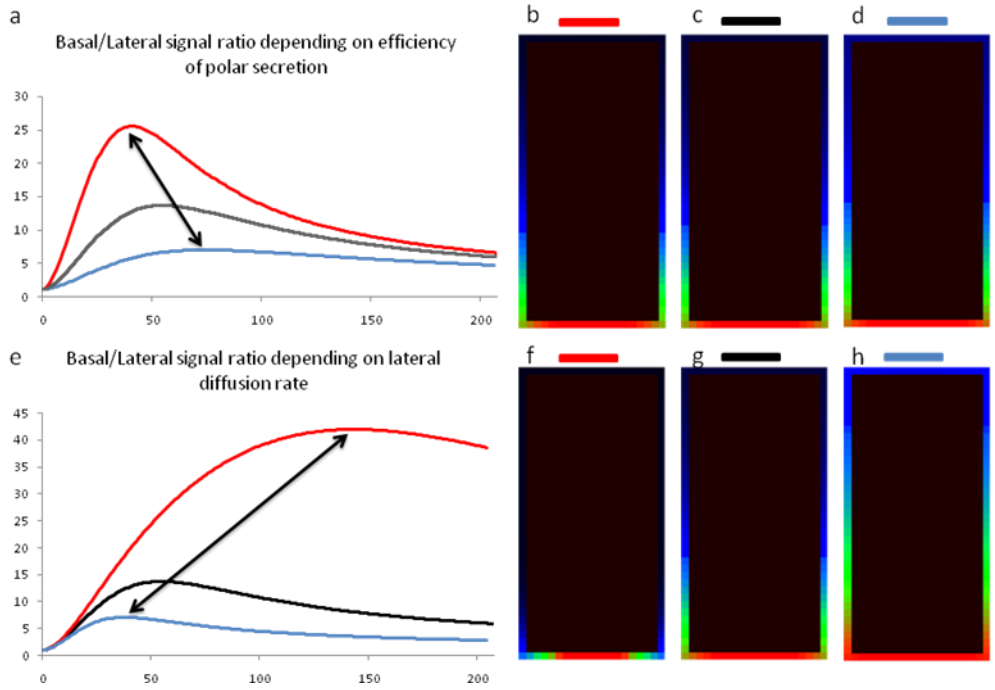
(*A-F*) Tracking FM4-64 uptake on PIN2-GFP (*A*) roots without (35 min DMSO / 10 min DMSO + FM4-64), and with 50 µM cycloheximide and energy inhibitors (-e), 0.02% sodium azide, 50mM 2-deoxy-D-glucose (*C and F*). The treatment with energy inhibitors combined with CHX largely stops the process of endocytosis what suggest efficient inhibition of other energy dependent processes like recycling and protein biosynthesis.



SFigure 3. Secretion pattern for inner-lateral localized BOR1 and apolar PIP2.

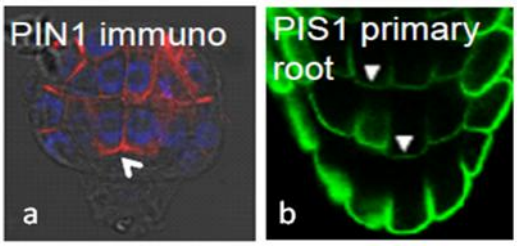
According to FRAP analysis BOR1 is specifically delivered to the inner polar domain (*E*), the recovery profile resembles the other tested markers except PIN2(*A*). The signal recovery profile suggest that the recovery is very low comparing to the pre-bleach signal. However, BOR1 as a sensitive protein to presence of boron, undergoes immediate

degradation, therefore the final signal recovery normalized by the signal depletion in neighboring nonbleached cells is quite high and reaches approximately 60% (*B and D*). PIP2 which is perceived as a nonpolar marker shows differential signal recovery at apical inner- and outer-lateral domain (*C and F*), what may be explained by different secretion activity or protein stability at different polar domains. Mean recovery profiles of all tested markers (*D*). Data are mean, n=4-8.



SFigure 4. Protein polar localisation depends on the efficiency of polar secretion and speed of protein lateral diffusion.

Computer simulations revealed that more concentrated secretion correlates with more polarized protein localization (*A-D*). Importantly, in case of lateral diffusion speed we have observed that, the lower diffusion speed the stronger protein concentration (*E-H*).



SFigure 5. Polar protein localization during plant development.

PIN1 basal localization in embryo stage 16/32 cells (*A*). PIS1-GFP polar protein localization, facing the environment at the columella primary root (*B*).

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Material and methods:

Plant material, growth conditions:

Plant material: PIN1::PIN1:GFP (Benkova et al, 2003), PIN2::PIN:GFP (Xu and Scheres, 2005), 35S::GFP:PIS1 (Růžička et al, 2010), PEN3::PEN3:GFP (Boutté et al 2007),

BOR1::BOR1:GFP (Takano et al, 2010) and 35S::PIP2:GFP (Cutler et al, 2000) PIN2::PIN1:GFP-2;eir1-1 (Wiśniewska et al, 2006).

Arabidopsis thaliana (L.) Heynh. seeds were sterilized with chlorine gas and stratified at 4°C for 2 days in the dark. Five-day-old seedlings were grown on vertically oriented plates containing *Arabidopsis* medium (AM; half-strength Murashige and Skoog medium, agar, 1% sucrose, pH 5.9) under a 16-h photoperiod, at 22°/18°C. For fluorescence recovery after photobleaching (FRAP) analysis, plants were grown vertically on the plate for 5 to 6 days, next placed in chambered cover glass (Nunc Lab-Tek), covered with slice of MS 1% sucrose solid medium and scanned as indicated. Due to the fact, that BOR1 gets degraded upon high boron conditions, BOR1-GFP line was tested using special boron deficient medium 0.3 μM boric acid (Takano et al, 2010).

Drug treatments:

In order to address the lateral diffusion rate, first we checked the energy inhibitor efficiency. We tested the endocytosis rate of treated and non treated seedlings by using FM4-64 endocytic tracer. The control seedlings were incubated for 10min in presence of 4 μM FM4-64 (Invitrogen), washed out and checked at the confocal microscope Zeiss 710. Seedlings treated with inhibitor, where initially pretreated 35min with 50 μM cyclocheximide (CHX; Sigma) and energy inhibitors (-e), 0.02% sodium azide, 50 mM 2-deoxy-D-glucose (Men et al, 2008) and 10min with CHX, -e and 4 μM FM4-64. All treatments were carried out in AM liquid medium on ice in the light and at least in triplicate, with a minimum of 12 roots for each treatment. Control treatments contained an equal amount of solvent (dimethylsulfoxide).

Immunolocalization:

Immunolocalizations where performed on embryos dissected from young and old ovules from 3 weeks old plants. The procedure was done by using Intavis in situ pro robot equipped with slides module (Sauer et al, 2006). The cell wall mutants where immunostained with anti-PIS1 antibodies using the same robot, however equipped with basket system, according to the published protocol (Sauer et al, 2006). Primary antibody was anti-PIN1 (Paciorek et al, 2005) 1:1000 and anti-PIS1 1:600 (Abas et al, 2006). Secondary antibody was Cy3 anti-rabbit (Sigma-Aldrich) 1:600.

Quantification of polarity:

The mean fluorescence signal intensity of different GFP-fussed lines at the polar and opposite or lateral sides of cells (as indicated Fig1.), where measured using Image J 1.40g (Rasband). This software provides the opportunity to draw lines of the same size along each of analyzed cell sides. Obtained mean pixel intensity values of certain length were then used to generate recovery curves and determine the polarity index—the ratio of X protein intensity at polar versus lateral or opposite sides.

Microscopy:

For confocal laser scanning microscopy, a Zeiss 710 with upright microscope stand and an Olympus fluoview FV10 with inverted microscope stand were used. Semi-quantitative confocal imaging was performed and analyze with Zeiss 710. Images were processed in Adobe Photoshop CS10 and assembled in Adobe Illustrator CS10 (Adobe Inc.). Fluorescence signal intensity was analyzed with Image J 1.40g (Rasband) and confocal software (Zeiss, Olympus). Data were statistically evaluated with Excel 2007 (Microsoft). All the 3D reconstructions where done using Zeiss 710 at 0,5µm interval size.

Total FRAP Analysis:

FRAP analysis was performed with a Zeiss 710 equipped with an inverted microscope, using a water-immersed 40× objective and 488-nm diode laser excitation. The cell of interest as well as the neighboring cells where bleached to avoid the influence of lateral diffusion from neighboring cells. Depending on the line different bleaching settings where made. For all of them we used 100% laser power and the bleaching was set to stop when the initial signal intensity will drop below 1%. The number of iterations was 200 for PIN1 which is expressed in the stele and 100 for all the markers localizing in epidermis. Postbleach scans were performed with 3% laser transmission and GFP emission was detected between 505 and 530 nm. The postbleach scans where performed every 30min until 180min, in case of PIN1 we performed additional scans in the early recovery stages as indicated (Fig3.). For analysis the FRAP recovery data we performed normalization basing on equation In=[(It-Imin)/(Imax-Imin)]*100; where In is the normalized intensity, It is the intensity at any time t, Imin is the minimum postphotobleaching intensity, and Imax is the mean pre-photobleaching intensity.

The obtained recovery value was than compared to initial signal intensity and increased by the percent of general signal depletion in non bleached neighboring cells.

Local FRAP analysis:

The 2-μm PM regions were bleached using 100% laser power and 80 iterations. The bleaching stopped when the signal dropped below 1% of initial intensity. First we measured the signal intensities at the prebleached region (ROI) and congruent (neighboring) region at the same domain (using Image J software which allows to draw the line of the same length and measure the mean signal intensity in two neighboring regions). Next, we bleached the ROI and subsequently measured the signal intensity at the ROI and

congruent region. In total, we registered 4 time points: 0min, B, 5min and 10min after bleaching. Postbleach scans were performed with 3% laser transmission and GFP emission was detected between 505 and 530 nm. For analysis of the FRAP data to compare relative protein mobility we normalized intensities by using the following equation: In=[(Imax-Imin)/(It-Imin)]; where In is the normalized intensity, It is the intensity at any time t, Imin is the minimum postphotobleaching intensity, and Imax is the mean pre-photobleaching intensity.

Polar Domain FRAP analysis:

The entire polar domains were bleached using 100% laser power and 100 iterations. The bleaching stopped when the signal dropped below 1% of initial intensity. Postbleach scans were performed with 3% laser transmission and GFP emission was detected between 505 and 530 nm. The postbleach scans where performed every 15min until 45min. To have clear answer whether localized recovery indeed occurred, we did 3D reconstructions of bleached cells at 0,5 µm interval size.

Protoplasting and Partial Degradation of the Cell Wall

A fresh protoplasting solution was prepared as following: 1.25% Cellulase (Yakult Farmaceutical Ind. Co., Ltd.), 0.3% Macerozyme (Yakult Farmaceutical Ind. Co., Ltd.), 0.4M D-Mannitol (Sigma), 20 mM MES monohydrate (Duchefa Biochimie) and 20 mM KCl (Merck). We adjusted the pH to 5.7 by using 1M TrisHCl (pH 7.5; Invitrogen). The solution was first warmed up for 10 minutes at 55oC, then cooled down at RT. 10 mM CaCl2 (Sigma) was added before use. For partial degradation of the cell wall we left out cellulose.

Computer model description:

We use the modification of our recent framework for maintenance of PIN polar domains in plant cells (Kleine-Vehn and Wabnik 2011). The apical, basal and neighboring lateral sides of root stele cell were modeled explicitly. For computational reasons, we represented the plasma membrane as a sequence of discrete membrane fragments each of 1x1 micron size. The lateral cell sides were considered a 2-fold longer than that of apical or basal cell sides to mimic geometry of root stele cells. The intracellular membranes were approximated by one single endosomal compartment that represented the common intracellular pool of PIN proteins (see Figures 1I, 4A and 6A). The redistribution of PIN proteins between membrane fragments and endosomal compartments was determined by the basis of the PIN turnover rates (k_{exo} and k_{endo}). This basal exo- and endocytosis rates are set to be constant for all cell sides. We considered that PIN proteins display lateral diffusion (D_m) within the plasma membrane. Scenario of preferential polar delivery of PIN proteins occurs to a central region within basal side of the cell, via endosomal trafficking mechanisms and subsequent spatially defined protein recycling. We modeled this process by assuming an increased rate of PIN delivery (k_{SPEX}) to the center of the basal polar domain. We considered two cases: (i) non-polar, *de novo* secretion of PIN proteins (k_{exo}), protein modification at the plasma membrane (m_{PIN}) and subsequent recycling and delivery of proteins to the polar domain (k_{endo}, k_{SPEX}). (ii) post-translational modification and polar secretion of PIN proteins (m_{PIN}, k_{SPEX}). The post-translational modification of PIN proteins was described by the following formula:

$$\frac{dPIN_i^{\mathrm{mod}}}{dt} = m_{\mathrm{mod}} \cdot PIN_i^{n\,\mathrm{mod}} - (1 - m_{\mathrm{mod}}) \cdot PIN_i^{\mathrm{mod}}$$

where PIN_i^{mod} is the pool of modified PINs in the *i*-th membrane fragment and PIN_i^{nmod} describes non-modified PIN pool (*de novo* synthesized proteins) . The parameter m_{mod} defines the rate of posttranslational modification of PIN proteins.

PIN proteins displayed specific distributions within the plasma membrane, namely recruitment into non-mobile plasma membrane microdomains (clusters). The non-mobile and mobile fractions of PINs in the membrane fragments were described by parameters f_1 and f_2, respectively. For the full description of the model we refer to our recent study (Kleine-Vehn and Wabnik et al., 2011).

Model parameters:

In the model simulations, the parameter values used were estimated from experimental data. From the fluorescent recovery after photobleaching (FRAP) experiments, the estimated non-mobile and mobile PIN fractions were $f_1 \sim 0.17$ and $f_2 \sim 0.84$, respectively. The parameter D_L was set to 1 $\mu m^2 s^{-1}$ by default. The basal PIN turnover rates were estimated for ~30 min half-time of PIN turnover and were $k_{exo} = k_{endo} = \ln(2)/T_{1/2} \sim 0.0005$ s^{-1}. We estimated the default rate of k_{SPEX} to 0.01 s^{-1}. Parameter D_m was set to ~0.1 $\mu m^2 s^{-1}$ by default. In control experiments, we varied parameters k_{SPEX} and D_m within one order of magnitude to predicts profiles of PIN protein distributions in the plasma membrane. In the simulations of pronounced PIN protein clustering we set f_1 *to* 0.83 and f_2 to 0.17, respectively.

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CHAPTER5

Concluding Remarks

Perspectives:

During this PhD project, the main focus was to gain a better understanding into processes that control polarity in plants. The identification of PIS1, an ABCG transporter, that decorates a thusfar novel polar domain (Chapter 2), triggered our interest in further investigating the concepts that underlie polarity establishment at different polar domains within plant cells. Although many tools, chemical and genetic, are available to manipulate PIN polarity. Most of these did not result in altered PIS1 localization (Chapter 3). To try to get a better understanding of how these different polarities are established we investigated protein dynamics at the respective domains (Chapter 4). This highlighted the importance of polar secretion, which is balanced by degradation. So far, we did not manage to gain insight into the regulatory molecular components of polar secretion.

To address this issue, it could be of interest to investigate other processes that display a prominent role for targeted secretion. One of the most prominent processes in which polarized secretion can be observed is tip growth as seen in growing pollen tubes and root hairs. Studying parallels between these tip growth and polar secretion in the root meristem seems highly interesting. Thusfar we performed a chemical screen on 260 compounds that efficiently disrupt pollen tube growth or germination (data not shown), however, none of these could alter PIS1 polarity. This suggests that polarity during tip growth processes are distinctly regulated from outer-polar secretion. Moreover, drugs that caused internal aggregates of PIS1 also displayed PIN aggregation, suggesting that these drugs disrupted very general trafficking processes, independent of the cargo. Alternatively, two other systems can be used to explore the polar secretion to the outer-lateral domain. The first is cytokinesis. This is the process during which a new cell wall is formed when a cell divides. This process is highly dynamic and requires a large amount of membranes within a short time-frame.

Therefore, all available secretory and recycled endosomes are recruited to the developing phragmoplast. Similarly, we observed that also PIS1 secretion becomes redirected to the phragmoplast during cytokinesis instead of to the outer-lateral domain. Studying how PIS1-secretory vesicles can be redirected in this process might give additional insight in how its secretion is regulated. The second is repolarisation of proteins during fungal invasion/infection. When a plant pathogenic fungus invades plant leaf epidermal cells, a haustorium is formed, to which several proteins are rapidly recruited. Among these proteins is PEN3, a close homolog of PIS1, which displays in root epidermal cells also an outer-lateral localization, suggesting that their trafficking is controlled by the same mechanism. The fact that fungal invasion can trigger repolarisation of PEN3 via hijacking the cell's secretory pathway, provides a potential entry point into better understanding PIS1 and PEN3 polarity.

During fungal invasion as well as cytokinesis a SNARE complexes are involved in closer of the vesicle to the membrane, tethering and vesicle fusion. Therefore, it will be of interest to screen the homologs of this machinery which decorate the outer-lateral domain and be functionally involved in polarized secretion of PIS1 and PEN3. While SNARE are involved in tethering and fusion of the correct vesicles, it is also essential to identify the motor proteins and actin filaments regulators that are involved in bringing the vesicles to that domain. It is known that actin is involved in secretion of apicaly, basaly and lateraly localized proteins PIN2, PIN1 and PIS1 respectively. Therefore a screen for components stabilizing and promoting actin polymerization could be of interest. Reveres genetic approch focused on actin related/binding proteins like ARP2/3 or WASP could give a glimpse of mechanistic specificity towards different polar domains. In order to do that T-DNA insertion mutant analysis combined with localization studies based on immunocytochemistry or epifluorescence should be performed. Taking into account the number of different actin genes expressed in the root meristem, it seems important to test actin filaments specificity in

different polar cargoes delivery. In that case also T-DNA insertion mutants analysis would be useful. In order to avoid lethality or ectopic expression issues inducible-dominant-negative lines should be established. To complete the analysis, the promoter swapping experiments should be performed in order to check the influence of expression on polar delivery.

Finally, an alternative approach would be to identify the domain(s) within PIS1, which are essential for its secretion to the outer-lateral domain. This could be done by creating chimeras between PIS1 and a related ABCG protein that does not show this typical polarity. At the protein level the great challenge would be to find the specific cargo sorting receptors recruiting the proteins and mediating their delivery to proper polar domain. Beside BP80 receptor which, resides at TGN and attributed with tyrosine motif determines the specific trafficking to PVC, non other sorting receptor is known. BP80 which is cycling between TGN, PVC and again TGN must have a specific signaling domain for targeting back to TGN. The others putative receptors mediating trafficking to other organelles or polar domains should have the same flag. Identification of these sequence or specific residues like tyrosine motif could help in selection of other receptors. In order to do that the directed mutagenesis approach could be used.

Author's Contribution:

Kamil Růžička, Lucia C. Strader, Aurélien Bailly, Haibing Yang, Joshua Blakeslee, Łukasz Łangowski, Eliška Nejedlá, Hironori Fujita, Hironori Itoh, Kunihiko Syōno, Jan Hejátko, William M. Gray, Enrico Martinoia, Markus Geisler, Bonnie Bartel, Angus S. Murphy, and Jiří Friml. *Arabidopsis PIS1* encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. (2010) PNAS vol. 107 no. 23 10749-10753.

Author's Contribution: ŁŁ performed experiments presented in Figure 1B-D, Figure 3A, SFigure 1A-C.

Łukasz Łangowski, Kamil Růžička, Satoshi Naramoto, Jürgen Kleine-Vehn and Jiří Friml. Trafficking to the outer polar domain defines the root-soil interface. (2010) PNAS 25;20(10):904-8

Author's Contribution: JF initiated the project; JF and ŁŁ designed the experiments; ŁŁ carried out most of the experiments; KR performed experiments presented in Figure 1G-J and Figure 2A; SN performed experiments presented in Figure 2B; JK performed experiments presented in Figure 1L; ŁŁ performed experiments presented in Figure 1A-F, K; Figure 3A-K, Figure 4D-J; Figure S1A-I; Figure S2A-K; Figure S3A-G; Figure S2A-F; ŁŁ associated the figures, ŁŁ and JF discussed the results and wrote the manuscript.

Łukasz Łangowski, Krzysztof Wabnik, Steffen Vanneste, Jiří Friml. Mechanistic framework for apical, basal and lateral polar localization in Arabidopsis.

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Publications:

Kleine-Vehn J, Wabnik K, Martinière A, **Łangowski Ł**, Willig K, Naramoto S, Leitner J, Tanaka H, Jakobs S, Robert S, Luschnig C, Govaerts W, Hell SW, Runions J, Friml J**. Mol Syst Biol.** 2011 Oct 25;7:540.

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Ruzicka K, Strader LC, Bailly A, Yang H, Blakeslee J, **Łangowski Ł**, Nejedlá E, Fujita H, Itoh H, Syono K, Hejátko J, Gray WM, Martinoia E, Geisler M, Bartel B, Murphy AS, Friml J. Arabidopsis PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. **Proc Natl Acad Sci U S A**. 2010 Jun 8;107(23):10749-53.

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